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The evaluation of functional feeds additives in commercial conditions with the pacific white leg shrimp

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THE EVALUATION OF FUNCTIONAL FEED ADDITIVES IN COMMERCIAL CONDITIONS WITH THE PACIFIC WHITE LEG SHRIMP (L. Vannamei)

by

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Ethical review statement

All experimental work involving animals complied with the Animal Scientific Procedure Act, all experimental work involving animals further complied with the Plymouth University Animal Welfare and Ethical Review Committee.

Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

This thesis has been proofread by a third party; no factural changes or additions or amendments to the argument were made as a result of this process. A copy of the thesis prior to proofreading will be made avalible to the examiners upon request.

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Publications

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ABSTRACT

THE EVALUATION OF FUNCTIONAL FEED ADDITIVES IN COMMERCIAL CONDITIONS WITH THE PACIFIC WHITE LEG SHRIMP (L. Vannamei).

Kurt Servin

Given the rapid expansion of aquaculture globally and with the *Litopenaeus vannamei* sector making a significant contribution to seafood production, there is an urgent need to address diet and feed formulation focused on sustainability and novel ingredients, without using chemotherapeutics. This thesis explores three bioactive natural agents used in shrimp diets to mitigate the threat of disease and confer a more resilient shrimp: (i) β-glucans and yeast-derived components as functional feed additives; (ii) a blend of phytobiotic herbal extractives and yeast (YAH); and (iii) Tuna Protein Hydrolysate (TLH) as growth-promoting and immunostimulants. All studies were conducted on juvenile shrimp in cages within an open system environment under high density with fluctuating, oxygen, temperature, salinity and photoperiod. Zootechnical parameters, and indices relating to haemocyte levels for immune competence and histological assessment of the intestine, with emphasis on gut morphology and integrity, were analysed. Also, the aim was to ascertain the effect of the functional feed additives on the gut microbiota associated with their potential modulatory capacity under commercial conditions. The findings confirmed that *β*-glucans and yeast combinations enhanced growth and survival of juvenile shrimp. For YAH, a 1% inclusion gave excellent improvements in growth and survival. For TLH, a 2% inclusion rate gave the best overall performance. These trials revealed a marked shift in the microbial enteric community with effects on phyla but more pronounced at the genera taxonomic level. The thesis discusses these aspects for securing a more efficient shrimp production industry with a cost-benefit assessment for economic appraisal of using function feed additives in this important aquaculture sector. The results are expected to be a comparative basis for other studies, and relevant for the aquaculture industry. The findings are expected to contribute to the advance of animal nutrition and health and for the increasing of shrimp farming industry.

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Work presented

- Servin, K., Tacon, A,G.J. (2018) The evaluation of exogenous enzymes under clear water, green water and bioflocs. In: Latin American & Caribbean Aquaculture 2018, Symposium. Bogota, Colombia. Oral presentation.

- Servin, K., Davies, S.J. (2018) Building the robust shrimp with the use of novel green tools. In: Congreso Nacional de Acuacultura, 2018, Symposium. Salon Figlos, Los Mochis, Sinaloa. Oral presentation.

- Servin, K., Merrifield, D., Tacon, A.G.J., Davies, S.J. (2018) Tuna Fish Hydrolysate in diets for white shrimp (*Litopenaeus vannamei*). In: Interntion Symposium of Fish Nutrition and Feeding, 2018. Las Palmas, Gran Canaria, Spain. Poster presentation.

- Servin, K., Lucien Brun, H., Chowdjury, M.A.K. (2017). Una revision de aplicacion de una protease en el alimento balanceado para optimizar los beneficios y la sostenibilidad de la cria de camaron *Penaeus Vannamei*. Latin American & Caribbean Aquaculture Society, 2017. Mazatlan International Center, Mazatlan, Sinaloa. Oral presentation.

- Servin, K. (2017) Evaluatino of functional feed additives with the use of floating cages under commercial conditions. In: Congreso Internacional Acuacultura de Camarón, 2017. Universidad La Salle, Obregon, Sonora, Mexico. Oral presentation.

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LIST OF ABREVIATIONS

ANF Antinutritional factor

BOD Biological oxygen demand

Da Dalton

DGGE Denaturating Gradient Gel Eletrophoresis

EMS Early Mortality Syndrome

FAO Food and Agriculture Organisation of the United Nations

FCR Feed conversion ratio

FFA Functional feed additive

FM Fish Meal

GM Gut microbiota

GS Gut sample

HDPE High density Poluehtylene

HPLC High-performance liquid chromatography

LAB Lactic acid bacteria

LefSe Linear discriminable analysis effect size

LDA Linear discriminable analysis

HTS High Throughput Sequencing

HPLC Peptidos

H&E Hematoxylin and eosin

MVD Microvilli diameter

MVH Microvilli height

MVL Microvilli lengh

NGS Next Generation Sequencing

nm Nanometres

OTUs Operational taxonomic units

PCoA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PL	Post larvae
$PL.m^{-1}$	Post larvae per meter
$PL.m^{-2}$	Post larvae per cubic meter
ppt	Parts per trillion
PVC	Polyvinyl chloride
RAS	Recirculating aquaculture systems
RDP	Ribosomal Database Project
ROI	Return of investment
QIIME	Quantitative Insights into Microbial Ecology
SD	Standard deviation
SE	Standard error
SFS	Shrimp fitness status*
SGR	Specific growth rate
SBM	Soybean Meal
TLH	Tuna liquid hydrolysate
THC	Total hemocyte counting
TPH	Tuna protein hydrolysate
YAH	Yeast and Herbs
W	Week
WG	Weight gain

CHAPTER 1: Introduction

1.1 Aquaculture an overview

Aquaculture is the fastest growing agricultural industry in the world; it has expanded globally with an increase of production with a growing list of species cultured in fresh and saltwater (Vetvicka et al., 2013). In particular, shrimp aquaculture sector has grown significantly for decades, but production has consistently been affected by serious problems linked to environmental degradation and both infectious and non-infectious diseases (Patil et al., 2011).

In between February to October 2013, the Mexican shrimp (*Litopenaeus vannamei*) usually known as Pacific white shrimp industry lost almost 70% of their production due to an Early Mortality Syndrome (EMS) outbreak, leaving the industry in a financial crisis. Control of disease is a priority, as shrimp production has to be ecologically and economically sustainable (Patil et al., 2011), losses in production of cultured shrimp have led to the realization that the goal in aquaculture is not merely to increase production but to make it sustainable and promoting good animal husbandry, high quality products, great reducing and combating disease.

With the growing demand for protein due to the expansion of global population to 9.7 billion by 2050 (UN, 2017), fish and shellfish are the most accessible animal protein, as well as a source of essential nutrients. The aquaculture industry has shown records growth historical records in recent years. In 2014, for example, aquaculture production

surpassed fisheries for the first time, and the production of high-value species, such as shrimp, is expected to continually increase in the next decade. Shrimp is the second most traded commodity in terms of value and, due to be a highly traded species, the supply of shrimp from farming has a significant influence on price trends. In 2008, the global shrimp farming accounted for 40% of all world production, with 60% of international trade being based on shrimp from farms.

The major farmed shrimp species is the *Litopenaeus vannamei*, commonly known as the whiteleg shrimp, the Pacific white shrimp or the king prawn. (FAO, 2008; FAO, 2016). Shrimp as part of the second largest subphylum on the planet has a tremendous relationship with the environment and large history with infectious disease.

The feeding strategies include a search for food in suspension, substrate scanning, and predation. Among the species with commercial appeal, the most prominent is the Penaeidae family, especially those of the genus *Litopenaeus*, such as the *Litopenaeus vannamei*. The Pacific white shrimp is endemic to the eastern Pacific coast, occurring naturally from Sonora, Mexico, to Tumbes, Peru. Nowadays, the species is considered pantropical, having its geographic distribution Throughput the entire tropical zone (Noga et al., 2006).

Opportunely, *L. vannamei* presents relevant zootechnical characteristics that make this species suitable for shrimp farming and, because of them, it is currently the most produced shrimp in the world. It can be stocked and produced even in small sizes; it

feeds satisfactorily even in captivity; it has excellent feed conversion; and fast and uniform growth. By the reason of being detritus and omnivore, it has a lower protein requirement than other species, requiring 20% to 35% protein in the diet (Jory & Cabrera, 2012). The growth and health in shrimp can be considered as energy reserves obtained by the organism and stored as corporal energy (Lemos & Phan, 2001), in addition to the capacity to absorb nutrients through natural productivity in the ponds and formulated diets.

The Pacific white shrimp adapts well to several environmental conditions and can be produced at high densities, such as 400 animals / m^2 in a controlled recirculation system. Regarding salinity, although they are considered marine shrimps, *L. vannamei* can also be produced in brackish water and freshwater. In China, up to 50% of the production is made in very low salinities, just as in Mexico, where the shrimp can be to 10% more expensive if it is produced in freshwater, due to a darker reddish body colour after cooking, a factor valued by the Mexican market.

1.1.1 Pacific white shrimp

Intensification of aquaculture systems and rapid growth in the number of production units has generated significant economic gains, an alternative protein source for the growing population of our planet but in turn has provided new opportunities for the emergence and transmission of aquatic pathogenic microorganisms for shrimp and other crustaceans (Bondad-Reantaso et al., 2005). The shrimp aquaculture sector has growth significantly for decades, but production has been affected by obstacles related to environmental degradation and both infections and non-infectious diseases (Patil et al., 2011).

Environment and sustainability fish by products are a sustainable and alternative ingredient, due to not only its lower price but also because of its relatively consistent composition and supply. On the other hand, plant proteins, such as oilseed cakes are often economically and nutritionally valuable sources of protein; however, potential problems associated with insufficient levels of essential amino acids (particularly lysine and methionine), antinutritional factors (ANF's), and poor palatability are the main concerns for feed formulators. Among plant proteins sources, soybean meal products are the most suitable sources to replace fish meal in aquatic feed, mainly because of their favorable protein levels as well as the amino acid profile that matches the animal requirement, except for the low level of methionine (Lim et al. 1998; Hardy 1999; Storebakken et al., 2000).

1.1.2 Global Shrimp farming and Mexico scenario

Currently, Mexico is in a recovering phase where farms are seeking solutions to the *Vibrio parahemolitucus,* the etiological agent of EMS, through preventive and management measures in aquaculture systems and technological developments. In parallel, the routine use of antibiotics must be reduced because of their adverse effect on the environment and the possibility of spreading antibiotic-resistant genes (Vetvicka et al., 2013). The demand for products free from chemicals and antibiotics is growing, which is increasing the potential market for immunostimulants, prebiotics, and probiotics.

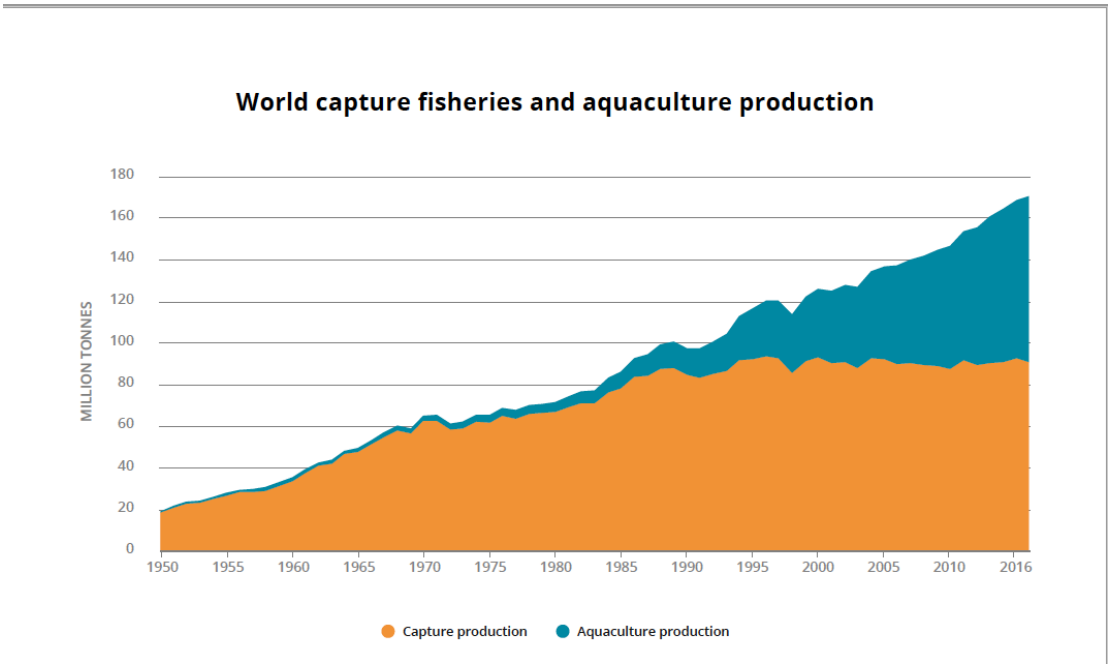


Figure 1.1: World capture fisheries and aquaculture production

Source: http://www.fao.org/documents/card/en/c/CA0190EN

1.2 Feed additives in shrimp diets

Among these, animal nutrition is closely related to the immunological resistance and adequate zootechnical parameters, with essential factors for animal health and performance. The term immunonutrition, for example, refers to the influence of nutrients to increase immune resistance and the modulation of metabolic processes, often associated with pathological conditions (Rogalsky and Martindale, 2019). In addition, food additives are substances included in food/feed with the aim at improving specific performances, such as weight gain, nutrient and energy utilization and health (Singh et al., 2015; Nunes et al., 2018).

The use of feed additives in shrimp culture is a common practice worldwide and widely accept by shrimp farmers, for both growth performance and/ or an immune enhancer, due to the animal's nutritional state and physiological status that can modulate the immune system accordingly and other related organic and systemic functions. Immunostimulants, including nutrients and non-nutritive substances, have been studied in recent years, and they are a natural compound that can modulate the immune system and improve growth and production (Zhang; Mai, 2014). Immunostimulants can be classified based on their sources, such as structural elements of bacteria, fungi, and yeast, seaweed, animal derived, hormones/cytokines, and synthetic products, and can be divided into nutritional, such as vitamins, minerals (both also called immunonutrients), and polyunsaturated fatty acids, and non-nutritional immunostimulant (Sohn et al., 2000; Sakai 1999). They can be provided as a

prophylactic treatment to overcome a possible immunosuppressive effect on the farming process under stress and super intensive shrimp production (Zhang; Mai, 2014).

These days, aquaculture aims to be eco-friendly, without the use of chemotherapeutics that may present negative aspects both for environment and animal health. Antibiotics can accumulate for a long period of time in the animals, making them unsuitable for human consumption. After the administration of enrofloxacin and ciprofloxacin, for example, it is necessary to wait 14 days to deplete all antibiotic residue from shrimp muscle, and another 14 days to disappear from shrimp hepatopancreas (Flores-Miranda et al., 2012). Thus, an eco-friendly method, such as the use of polysaccharides or medicinal herbs as prophylactic agents and as feed additives may benefit the quality of shrimp farming, especially in anticipation of disease outbreaks (Karunasagar et. al 2014).

Feed tractability particularly for shrimps and palatability is closely related to the presence of attractive chemical compounds, normally associated with shrimp´s prey components under wild conditions. Thus, nutritional and organoleptically accepted diets are essential to achieving satisfactory intake and performance. For crustaceans, feed with high attractability are those with low molecular weight, soluble in water and ethanol, and related to potentials prey constituents. These include compounds such as free amino acids, especially taurine, hydroxyproline, glycine, organic acids, nucleotides, and nucleosides, *β*ine, and small peptides (Tantikitti et al 2013).

A major important factor determining the success of the feed acceptance is the palatability and the attractiveness of the feed, which promotes high ingestion and leads to better utilization of available nutrients (Derby; Sorensen, 2008; Glencross; Smith, 2014). Diet palatability will help to reduce the time that shrimp spend approaching the feed and it will limit nutrient leaching and feed loss, which will reduce deterioration of the rearing pond environment from overloaded nutrient input (Hardy 2010). As well as good diet palatability, environmental parameters like pH and salinity have pronounced influence on the chemoreception and feeding response in the shrimps, in particular diets are more chemotactically at a pH between 7.0 - 9.0 and a salinity between 15 and 25 ppt. The shrimp feed intake was observed to decline by 50% at pH 6.0 and 10 (Fernandez et al 1995).

1.3 Immunostimulants in aquaculture

An immunostimulant can be defined as substances which enhances the innate or non-specific immune response by interacting directly with cells of the immune system and activating them (Mastan, 2015). Immunostimulants are mainly a group including carbohydrate, products of fermentation, bacterial preparations, polysaccharides, animal or plant extracts and nutritional factor or cytokines (Barman et al., 2013).

Immunostimulants are considered as a useful tool for enhancing immune status of cultured organisms (Meena et al., 2011). Among the different immunostimulants used

in aquaculture, β-glucans have become one of the most commonly used. The dietary administration of β-glucans has been reported to enhance many types of immune responses, resistance to bacterial and viral infections and environmental stress in many farmed species (Vetvicka et al., 2013).

A number of immunostimulants have been reported to be effective in enhancing non-specific immunological supplements leading to diseases resistance of fish and crustaceans (Aspines-Amar et al., 2015). This is particularly important in species that are raised in environments where the nature of pathogens is unknown, and so immunization by a specific vaccine is not practical (Maqsood et al.).

Immunostimulants do not generate a specific response to certain pathogens but cause an overall response that hastens recognition and perish of a broad range of infectious agents and foreign substances (Campos et al., 1993; Secombes, 1994; Sordello et al., 1997). In contrast to the immune system of a fish that is capable of developing and mounting acquired immunological response, most invertebrates including shrimp lack an antibody-based type adaptive immune system (Aspines-Amar et al., 2015). Therefore, specific immunomodulation and immunity via the use of vaccinations is not viable for shrimp. In invertebrates, the non-specific response is considered the first line of defense against invading pathogens and is the single immunological mechanism of protection from disease (Barman et al., 2013). In shrimp, the non-specific defense system includes both cellular and humoral components as shown in Table 1.1 (Martinez, 2007).

Table 1.1: Nonspecific immune system of shrimp, with cellular and humoral components of crustacean immune system (Martinez, 2007).

Cellular components	Humoral components
Phagocytosis	Anticogulant proteins
Encapsulation	Agglutinins
Formation of nodules	Phenoloxidase enzyme
	Antimicrobial peptides
	Free Radicals

Cellular defence components include all reactions performed directly by hemocytes, like phagocytosis, encapsulation, nodule formation and number of other mechanisms. While, humoral components include the activation and release of molecules stored within hemocytes (anticoagulant proteins, agglutinins, antimicrobial peptides, protease inhibitors, etc.) (Martinez, 2013: Jiravanichpaisal et al., 2006).

In shrimp the mechanism of action against invasive pathogens is quite simple with an open circulatory system based on hemolymph composed of plasma and hemocytes. The first line of defense is the exoskeleton or cuticle which coves the external body surface including hind duct and reproductive duct, this limits the entry of bacteria, parasites and microorganisms, nevertheless the first entry point for many pathogens is the digestive tract or shrimp gut where, a mixture of bacteria associated and biochemical reactions neutralizes and destroys invaders (Jiravanichpaisal, 2013). Once the pathogen has crossed the outer defense barriers, hemocytes play a key role in the shrimp immune response (Martinez, 2007), Hemocytes participate in the inactivation of invading organisms and are also involved in the regulation of different physiological

functions (exoskeleton hardening, cuticle damage healing, coagulation etc.) (Jiravanichpaisal, 2006).

The immune recognition of pathogens is carried out by the hemocytes with molecules that are capable of recognizing structures from the cell walls from invader organism such as proteins and β-1-3 glucans, 3, lipopolysaccharides and peptidoglycans (Lin et, al.2006: Vargas – Albores 2000). Once invading organisms are detected, hemocytes become activated then a whole cascade of mechanisms is triggered to control or remove foreign agents (Martinez, 2007). These include Phenoloxidase activity, free radicals and antioxidant generation, phagocytosis, and encapsulation and nodule formation.

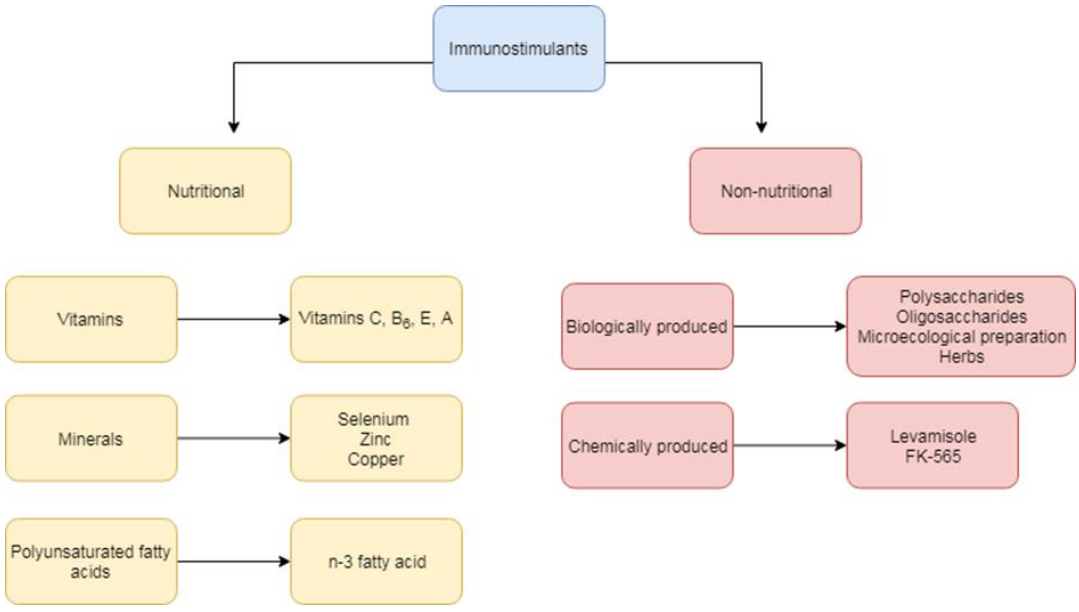


Figure 1.2: Classification of various immunostimulants used in commercial shrimp farming and examples. Data from Zhang and Mai, 2014.

Immunostimulants are chemicals, drugs, stressors, or any action that can improve the non-specific defence mechanisms, and they can be classified into two classes, namely nutritional and non-nutritional and (Figure 1.1). They different from probiotics, in that they are not living cells. Many molecules that can be classified as immunostimulants such as glucans, lipopolysaccharides, and peptidoglycans are also understood as PAMPs (Karunasagar et al., 2014).

The most used and successful supplements that promote higher survival of crustaceans against infectious diseases are (1) live bacteria; (1) killed bacteria (bacterins or bacterial antigen); (3) glucan extracts from yeast; (4) peptidoglycan preparations from non-pathogenic Gram-positive bacteria; and (5) lipopolysaccharides (LPS) from Gram-negative bacteria. It is also possible to cite other compounds such as (6) commercial algal extracts, (7) herbs and spices mixtures, (8) macro-algae derived alginates, (9) vitamins and antioxidants, and (10) probiotic cultures (Smith et al., 2014).

Some of the pathogens that have been controlled successfully by using immunostimulants in fish and shrimp include *Vibrio parahaemolyticus*, *V. harvey*i, *V. alginolyticus*, yellow head virus (YHD), white spot disease (WSSV), and *Ichthyophthirius multifiliis* (Mastan et al., 2015). There are three methods to administer immunostimulants in aquaculture such as injection, immersion and oral uptake. Injection and immersion require handling of the organisms or confining them in a small area during the application, and these methods are laborious, time-consuming and stressful. On the other hand, oral administration is a non-stressful approach which can

be used within any size of independently feeding fish/shrimp. This approach is commonly used in extensive aquaculture systems.

For the practical use of immunostimulants, the timing, dosages, method of administration and health status of the organisms must be taken into consideration (Mastan et al., 2015) and it is important to mention that the efficiency of using an immunostimulant is usually determined by the strategy by which it is applied such as stage, dosage and sequence (Bricknell & Delmo et al., 2005).

The effects of immunostimulants on non-specific immune systems are generally of short duration, and the continuous use of an immunostimulant may up-regulate the immune system and maintain this status until the immunostimulant is withdrawn, or it may cause adverse effects such as tolerance or immunosuppression (Aspones-Amar et al., 2015).

Aspones-Amar et al., 2015 have stated the importance in defining the specific dosage rates and efficacy of the immunostimulant when applying, pulse administration, oscillation, (administering immunostimulant-supplemented and non-supplemented diets alternately) which raises the immune response from a resting level to a heightened response. This has been shown to be a better strategy of immunostimulant application.

In addition to the enhancement of the non-specific defense mechanism and the increment of the resistance to specific pathogens, some immunostimulants also have shown growth-promoting activity. Growth enhancement could result from improved disease resistance (Aspines-Amar et al., 2015).

Some issues that have been detected in the use of immunostimulants in shrimp culture is the lack of correlation between *in vitro* and *in vivo* studies. Consequently, doses determined *in vitro* cannot be directly extrapolated to large-scale production systems (Aspines-Amar et al., 2015). Therefore, the immunostimulants may end up being administrated at high doses or for too long resulting in overstimulation and exhaustion of the immune system (Aspines-Amar et al., 2015). There are few long-term studies conducted in large-scale production units and most of them were executed in Asian farms, so it is critical to generate information under local conditions with the aim of finding the optimal doses and treatment time.

1.4 The gut microbiota

The superficial intestinal epithelial villa formed during early life stages of invertebrates including crustacea are colonized by a great number of different microorganisms. These develop commensal relations with their intestinal host organ; this particular group of microorganisms is called microbiota (Harris, 1993; Daffonchio et al., 2016). Understanding the role of the intestinal microbiota and its relationship with the host is essential to better comprehend how the gut microbiota can interfere in the host health

status, as well as in the host digestive process. Different studies focus on how to modulate the composition and metabolic function of these commensal microbial communities. Numerous strategies have been developed to adjust the gut microbiota, allowing the intestinal colonization with beneficial bacteria, through the rearing water or dietary inclusion of prebiotics, probiotics, and symbiotics, with positive results in term of growth and feed efficiency (Castex et al., 2014).

The gut microbiota has been described as the “new organ” (O´Hara et al., 2006) and has a crucial role in digestion, nutrition, and immune response (McFall-Ngai, 2002). Moreover, intestinal microbiota may protect against gastrointestinal infections (Merrifield et al., 2014), and against pathogenic microorganisms, through competition for nutrients and immunostimulation of enteric cells (Brown; Sadarangani; Finlay, 2013). However, it is worth remembering that shrimp intestine is coated by the peritrophic membrane, which is semipermeable and acellular. This membrane, which lines the medium gut, isolates the intestinal contents of the epithelium, making gut colonization on this animal more difficult (Karunasagar et al., 2014). Microorganisms capable of colonizing the shrimp digestive tract for a prolonged period relies mainly on the fact that the intestine remains intact during molting (Soonthornchai et al., 2015).

1.4.1 Indigenous gut microbiota

The intestinal microbiota of shrimp is also influenced by its complex life stages. Post-larvae and juveniles up to one-month-old tend to present Comamonadacea (*B*proteobacteria class) as the most dominant bacteria. On the other hand, Flavobacteriaceae (Bacteoidetes) are the most commonly found in two month old juveniles. Finally, the dominant bacteria in juveniles aged three months are the Vibrionaceae (Classe Gammaproteobacteria) (Huang et al., 2016). Besides this fact, it should be pointed out that the most common bacteria in the intestine of shrimp regardless of despite life stages, are Gammaproteobacteria Vibrionales, followed by Alphaproteobacteria Rhodobacteraceae, Chloroplast Stramenopiles, and Bacteroidia Bacteroidales (Cardona et al., 2016). Additionally, Lakshmi, Viswanath, and Gopal (2013) cited as indigenous predominant intestinal. (microbiota of shrimp Vibrio and Pseudomona). The authors highlighted that the lactic acid bacteria are often subdominant, especially the genus *Carnobacter*.

Apart from changing with age, the shrimp gut microbiota tends to adapt and be influenced by drugs, feed, physiological aspects of the host, and environment (Xiong; Zhu; Zhang, 2014; He et al., 2017; Huang et al., 2018). However, there is no consensus on Specifically, about the effect of the environment on shrimp gut microbiota. According to Cardona et al. (2016), the shrimp intestinal microbiota is, in fact, influenced by the surrounding environment. According to these authors, the microbiota of shrimp raised in cristal clearwater reflects the local marine water

bacterial community. Similarity shrimp produced in biofloc technology, will have their intestinal microbiota influenced by the physical variations that occur in the production system (tank, ponds, raceways) such as fluctuations in chlorophyll, pH, nitrogen, and others. In contrast, Tzeng et al. (2015) concluded that shrimp genetic has a greater influence on the composition of the intestinal microbiota than the habitat.

Regarding the main bacteria used as probiotic in shrimp farming, it is certain to say that the most common bacteria groups belong to the phyla *Firmicutes* and *Proteobacteria*, with the *Gammaproteobacterial being* the most common from the last phylum. In addition, the lactic acid bacteria now are studied as a possible probiotic for crustaceans.

1.4.2 Probiotics

Regarding the main bacteria used as probiotic in shrimp farming, it is certain to say that the most common bacteria groups belong to the phyla *Firmicutes* and *Proteobacteria*, with the *Gammaproteobacterial being* the most common from the last phylum. In addition, the lactic acid bacteria now are studied as a possible probiotic for crustaceans.
Table 1.2 summarizes the main bacteria probiotics used for shrimp. It is important to differentiate probiotic from prebiotic. Probiotics are innocuous microorganisms that compete with pathogenic ones and thereby exclude them and promote the balance of microbiota. Prebiotics, on the other hand, are substances that stimulate the growth and activity of probiotic microorganisms.

Table 1.2: The main probiotic bacteria currently used in shrimp farming.

Phylum / Class	Genus / Species	Source
Actinobacteria	*Athrobacter* sp	
	Micrococcus sp	
Gammaproteobacteria	*Alteromonas* sp	
	Halomonas sp	
	Pseudomonas spp	Castex; Daniels; Chim, 2014
	Shewanella spp	
	Vibrio spp	
Deltaproteobacteria	*Aeromonas* sp	
	Bdellovibrio sp	
Firmicutes	*Bacillus* spp	
	Bacillus vireti *	Hindu et al. 2018
	Carnobacterium spp	Castex; Daniels; Chim, 2014
	Exiguibacterium arabatum	Congo et al. 2017
	Lactobacillus spp	Castex; Daniels; Chim, 2014
	Lactobacillus plantarum *	Vieira et al. 2008
	Lactococcus spp	Castex; Daniels; Chim, 2014
	Lactococcus lactis	
	Leuconostoc mesenteroides	Merrifield et al. 2014
	Pediococcus acidilactici	Castex; Daniels; Chim, 2014
	Pediococcus pentosaceus *	Leyva-Madrigal et al. 2011
	Staphylococcus haemolyticus *	
	Streptococcus faecalis	Merrifield et al. 2014

* Bacteria with antagonist actions against pathogens.

1.4.3. Intestinal microbiota and nutrition

Feed additives may also be used to influence animal intestinal microbiota and they can be a great tool to improve animal health and production. For example, the use of protein hydrolysates in aquaculture is well established in the nutrition of fish (Kolkovski; Czesny; Dabrowski, 2000; Refstie et al., 2004; Martinez-Alvarez; Chamorro; Breves, 2015). For aquaculture, the search to include sustainable feed ingredients for aquafeeds could lead to the stabilization of potentially beneficial bacteria in the animal gut. Furthermore, the employment of novel aquafeed additives may be a meaningful instrument to promote animal health and gut microbiota balance (Li et al. 2018). Thus, it is crucial to better understand the patterns in gut bacteria colonization and modulation, in addition, to identifying the biological potential and environmental aspects in the composition of gut microbiota in cultured shrimps, and how they can be related to feeding additives such as Tuna Liquid Hydrolysate (TLH). For this, the use of appropriate techniques such as clone library analysis, denaturing gradient gel electrophoresis (DGGE), and next-generation sequencing (NGS) may facilitate a deeper understanding of the gut microbiota (Liu et al., 2011; Huang et a., 2016).

Despite the efforts to study the shrimp gut microbiota, little is known about the role of novel feed additives on its function and nutritional dynamics, such as hydrolysates from tuna and their effect remodelling the gut microbiota and the nonspecific immune response. Therefore, the main purpose of the present research trial was to assess the effect of TLH on shrimp performance and gut health, in terms of growth performance,

feed acceptance, and gut microbiota modulation and morphology, on animals raised in an intensive commercial system. Gut microbiota changes as shrimp age and tends to adapt to environmental conditions and can be influenced by drugs, feed and environment.

Changes in gut morphology from shrimps with the inclusion of functional feed additives in shrimp diets has significant impact on the reduction of colonization of pathogenetic bacteria in the gut (Hoseinifar et al. 2019). It has been reported that the use of FFA can modify the gut structure in numerous species with the inclusion of TLH (Siddik et al. 2018). Some of the evidence is associated with an increase in superficial structures where absorption of nutrients takes place in the intestine.

1.5 Yeast and β-glucans in aquaculture

For the last decade, there has been a lot of work around the implementation of natural additives (i.e natural prophylactic substances used for growth, promoting, animal health and to substitute antibiotics) to boost the immune system of shrimps in aquaculture using natural products such as ginger, garlic, yeast, herbs, and β-glucans (Emeka et al. 2014)

Yeasts are unicellular eukaryotic microorganism that are taxonomically placed within the phyla Ascomycota and Basidiomycota within the Kingdom Fungi. They are widely distributed in several natural environments such as soil, freshwater, and seawater. Their number and species distributions are dependent on the concentrations and types

of available organic materials. It has been proven that several yeast compounds have significant biological value as reagents, cell proteins, vitamins, pigments and enzymes (Navarrete et al., 2014).

Yeast glucans (β 1-3 and β1-6 linked glucan) and β-1,3 glucan (VST) are derived from cell walls of baker´s yeast like *Saccharomyces cerevisiae* and *Schizophyllum commune*, respectively (Barman et al., 2013). As shown in Figure 1.3 the yeast cell wall is composed of complex polymers of β-glucan (1,3) and (1,6), mannan-oligosaccharide (MOS) and chitin. MOS is located on the surface of the cell wall (Anward et al. 2017). Meanwhile, yeast glucans are often present in the inner wall layer and are associated with other cell components such as chitin.

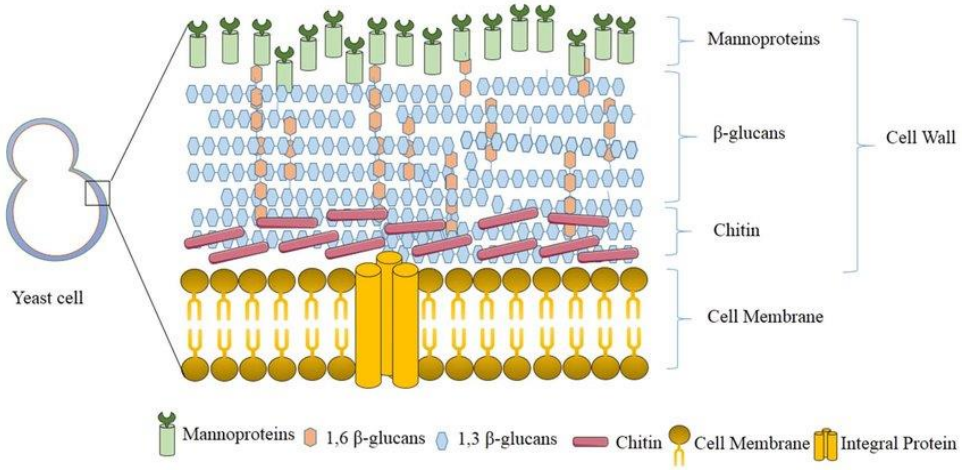


Figure 1.3. Yeast cell wall[1].

[1] Anwar et al., 2017. A review of β-glucans as a growth promoter and antibiotic alternative against enteric pathogens in poultry. https://doi.org/10.1017/S0043933917000241

1.5.1 β-glucan

β-glucans are a heterogeneous group of glucose polymers, consisting of a backbone of *B* linked *B*-D-glucopyranosyl units with *B* (1,6) linked side chains of different distribution and length (Dharmendra Kumar et al., 2016). The structure of the β-glucans depends on the source. The yeast β-glucan has *B* (1,6) branches further with new *B* (1,3) regions (Akramiene et al., 2007). Among the different sources of β-glucan in nature, yeast β-glucan has been the most widely used in aquaculture as a functional dietary supplement.

Among different immunostimulants used in aquaculture, β-glucan is one of the most promising. In nature, β-glucans are widespread in the cell wall of many plants (wheat, barley, and oat), baker´s and brewer's yeast (*Saccharomyces* genus) and Echinacea members (Kumar et al., 2013).

Many immunostimulants employed in aquafeed are polysaccharides derived from bacteria, fungi or yeast, and plants. β-glucans are polysaccharides commonly considered for use in aquafeeds due to their natural occurrence and no residue occurrence in animal tissues or by-products (Zhang; Mai, 2014). β-glucans are a heterogeneous group of glucose polymers, being the most relevant the β-1,3 and β 1,6

glucan. They are most commonly found in the cell wall of yeast, fungi, or cereal plants, although they can be also found in some species of seaweed and mushrooms (Meena et al., 2012).

The use of β-glucan in aquaculture has been assessed for years in several species of aquatic organisms under different conditions; however, most experiments have been performed in laboratories that are far from simulating actual conditions faced in commercial shrimp farms. Most of the β-glucan studies are carried out for at least 4-6 weeks, which is a fraction of the actual shrimp farming cycle, which is usually 15 to 25 weeks duration to reach commercial size. Limitations related to commercial trials involved, environmental fluctuations, electricity shortage, feed dispersal, shrimp loosses, complicated management and miscalculations in general are part of the commercial trials.

One of the benefits of field-testing is the extrapolation of the data obtained during the trials to the balanced feed industry because the information gathered is more approximated to the market conditions than lab studies.

Despite the existing knowledge of β-glucans in aquaculture in Mexico, (Nieves-Rodríguez et al. 2018), the use in the feed industry is not standard for several reasons, mainly the cost-benefit ratio. Most publications related to the use of β-glucan in aquaculture are based in Asian farming conditions and do not demonstrate the potential use of mean values. The present study was carried out to investigate the

effects of β-glucans in shrimp farming under commercial conditions evaluating shrimp growth and composition to observe the performance and benefits of these products in actual farming conditions including an economic analysis reflecting the potential of β-glucan in shrimp aquaculture in Mexico and Latin America.

The use of β-glucan on shrimp feeds has been around the industry for at least twenty years and the enhancement of *vibrio sp* resistance by supplementations of diets (Cruz, 1999) is well documented, the level of inclusion can range from 0.2 to 0.1 kg per ton of feed is the regular inclusion rate in commercial shrimp feeds, nevertheless the quality, concentration, source and prices can be very dissimilar, also the extraction process and equipment which my result into a large number of variables when evaluating β-glucan with aquatic animals.

1.6 Yeast and Herbs

Herbs immunostimulant actions are based on their botanical's compounds, they are exclusively derived from plants and can be understood as allopathic therapeutic. Homeopathy treatment is based on infinite or dynamited dilution, using not exclusively plant-derived substances, such as minerals, and it is considerate holistic medicine (Heinrich et al., 2012; Moreira et al. 2014).

Chinese and Indian herbs (i.e. plant valued for its medicinal properties) are non-nutritional immunostimulants biologically produced, which have been used as

traditional human medicines and immune booster for thousands of years (Zhang; Mai, 2014). By their secondary metabolism, plants synthesize several active compounds, which have antimicrobial and anthelmintic activities, as well as immunomodulatory properties (Sakai, 1999; Chakraborty and Hancz, 2011). The therapeutic potential and immunostimulant action of herbs are due to the high diversity of active botanicals compounds, such as polysaccharides, polypeptides, organic acids, alkaloids, glycosides, saponins, tannins, phenolic compounds, flavonoids, volatile oils, among others. Additionally, these compounds may present different mechanisms of action (Zhang, Mai, 2014; Veseeharan, Thaya, 2014). The diversity of active compounds added to the occurrence of synergistic effects decrease the probability of selection of resistant pathogens. Moreover, the use of herbs has advantages such as lower toxicity [2] in comparison to pharmacologically active synthetic drugs and the high potential for biodegradation, which results in the low or absence of chemical residues in the water and in the meat or its sub-products. Nevertheless, although herbs are widely used and assumed to be safe, they can be toxic. Usually, a misidentification of the plant or incorrectly preparation and administration are reported in cases of poisoning (Karimi et al. 2015). In financial terms, they tend to be inexpensive (Sakai, 1999; Veseeharan, Thaya, 2014).

[2] Although herbs are widely used and assumed to be safe, they can be toxic. Usually, a misidentification of the plant or incorrectly preparation and administration are reported in cases of poisoning. Karimi et al. 2015. J Nephropharmacol. 2015; 4(1): 27–30.

Finally, it is important to highlight that herbs and phytobiotics, whether as a feed additive or phytomedicines, are totally different to and not correlated with homeopathy.

There are still some questions regarding absorption and the mode of action of the immunostimulants in crustaceans. However, despite some questions and without a total agreement between studies, their acceptance by the farmers is currently very high, and the most recent research reveals an encouraging performance. Sarlin and Philip (2011), in a comparative study, reported that the marine yeasts *Debaryomyces hansenii* and *Candida tropicalis* may act as an immunostimulant in the Indian white prawn *Fenneropenaeus indicus*. These latter workers investigated the efficacy of these two species of marine yeast, in comparison to baker´s yeast *Saccharomyces cerevisiae* on the disease resistance and immune response of *F. indicus* challenged with the white spot syndrome virus (WSSV). The study showed that the marine yeast diet is an effective immunostimulant for *F. Indicus*, performing even better than baker's yeast.

Flores-Miranda et al. (2011) evaluated the effect of microbial immunostimulants on the survival and immune response of juvenile *Litopenaeus vannamei* challenged with *Vibrio sinaloensis* strains. A combination of four lactic acid bacteria (LAB), one yeast, and attractant oil was tested. The results indicated that these microbial immunostimulants administered every three days is a satisfactory feed additive against *Vibrio* spp. in shrimp culture, on both survival rate and immune parameters. In addition, Genio et al. (2014) showed that a dietary administration of crude lipopolysaccharide from *Vibrio*

harvey enhanced the resistance of the tiger shrimp *Penaeus monodon* postlarvae, against WSSV infection.

Bacteria, probiotics have been reported to impact growth rate, disease resistance, and survival rate in a number of studies. Far et al. (2009) demonstrated the beneficial effect of *Bacillus subtilis* on growth performance and survival of *Litopenaeus vannamei*. Nimrat; Boonthainb; Vuthiphandchai (2011) stated the favourable effect of different probiotic organisms and their combinations on the rearing of shrimps. Kongnum; Hongpattarakere (2012) observed that *Lactobacillus plantarum*, when added in shrimp feed, enhanced animal growth and survival, after challenging with *Vibrio harveyi*; authors also isolated LAB with inhibitory activity against *V. harveyi* from shrimp intestines. Venkat; Sahu; Jain (2004) used *Lactobacillus*-based diet to improve growth of *Macrobrachium rosenbergii.* Finally, Wang et al (2012) showed that the dietary supplementation of both viable and dead probiotics - but especially viable, can improve growth and survival rates of white shrimps.

Another type of feed additive, Bermuda grass (*Cynodon dactylon)* extracts*,* have also been deomonstrated to improve shrimp disease resistance and growth performance was investigated by Balasubramanian et al. (2008). These investigators examined the antiviral activity of a large scale produced plant extract of *Cynodon dactylon*, administrated through oral route to *Penaeus monodon*, against the WSSV*, in vivo*. The results of the study showed that the plant extract of *C. dactylon* was found to be highly effective in preventing WSSV infection. Immanuel et al. (2004) demonstrated that

survival, growth and pathogen load (*Vibrio parahaemolyticus*) on juveniles *P. indicus* were improved by adding butanolic extracts form terrestrial herbs and seaweeds. Citarasu et al. (2006) investigated the influenced of five selected Indian immunostimulant herbs against WSSV infection in *P. monodon,* with reference to hematological, biochemical and immunological changes. The work revealed that the application of herbal immunostimulants can be effective against shrimp viral pathogenesis and they can be recommended for shrimp culture. Table 1.3 summarizes the most common used in shrimp farming.

Despite the efforts to study the shrimp microbiota, little is known about the role of β-glucans, herbal blends and fish hydrolysates additives and their effect in remodeling the gut microbiota and producing a nonspecific immune response. Therefore, the aim of this study was to investigate the ability of a commercial immunostimulant, namely the functional feed additives, to improve growth, performance, and survival of farmed shrimp *Litopeaneus vananmei*, with a more comprehensive analysis in the modulation of the gut microbiota under controlled experimental conditions. The study evaluated growth performance, feed utilization efficiency and several physiological and health stats in addition to gut integrity and microbial ecology.

The feed attractability and palatability is crucial to shrimp performance in aquaculture. Shrimp may show selective feeding (Suresh; Varagam; Nates, 2011), thus to achieve satisfactory intakes, resulting in a successful shrimp farming, it is necessary that

palatability studies are carried out, and that the economic impact of the new feed additive be correctly measured.

Table 1.3: Examples of common immunostimulants and their effects in shrimp.

Immunostimulant	Example	Effects	Source
Bacterial endotoxin (LPS)	Crude lipopolysaccharide	Improved resistance against WSSV	Genio et al. (2014)
	Lipopolysaccharide	Higher granular and semi-granular haemocyte response	Xian et al (2017)
Killed bacteria	Vibrio bacterin	Improved immunity, less anatomical deformities, and better productivity	Ray et al. (2017)
Peptidoglycan preparations	Peptidoglycan	Promotion of muscle growth and enhancement of digestive proteases activities	Pan et a. (2015)
		Enhanced phagocytic activity and improved resistance against WSSV	Itami et al. (1998)
Macro-algae alginates	Alginates-derived oligosaccharides	Increased immuno functions and resistance against *Vibrio harveyi*	Jiang et al. (2017)
Micro-algae polysaccharide	Fucoidan	Improved defence against *Vibrio alginolyticus* and WSSV	Chen et al. (2016)
		Better survival and reduced impact of WSSV infection	Chotigeat et al. (2004)
Glucose polymers	β-glucans	Higher immunity and WSSV resistance	Bai et al. (2014)

		Improved resistance against white muscle disease	Pavadi et al. (2018)
Herbs	Chinese herbs	Enhanced immunity and disease resistance	Yeh et al. (2008)
		Improved immune parameters and resistance against WSSV	Chang et al. (2017)
	Indian herbs	Improved resistance against WSSV	Citarasu et al. (2006)
	Mixture of herbs	Antibacterial function, and improved growth and survival	AftaUddin et al. (2017)

LPS: Lipopolysaccharide; WSSV: White Spot Syndrome Virus

1.7 Peptides and Tuna Liquid Hydrolizates

The inclusion of TLH in shrimp diets has been reported previously to have significant impact on shrimp survival and growtht (Nguyen et al. 2012). It has been stated that the use of TLH can modify the gut structure in numerous species with the inclusion of TLH (Siddik et al. 2018). Some of the evidence is associated with an increase in superficial structures where absorption of nutrients happens in the intestine.

Antimicrobial peptides (AMPs) are a major component of the innate immune defense response system in marine invertebrates (Tincu & Taylor 2004). In crustaceans, there have been a number of studies to comprehend the AMP defence mechanisms. In the year 1972, bacterial activities were observed in the lobster *Homarus americanus spp* plasma and hepatopancreas. The bloodstream of crustaceans is mediated by the

hemocytes, and the hypothesis that AMPs play on important role in defence has been supported by the isolation of several peptide displaying antimicrobial activity from crabs and shrimps (Tincu & Taylor 2004). They are defined as molecules less than 10k Da in mass and of varying molecular weight which shows antimicrobial properties, (Boman 1995) and provides immediate and rapid response against invading harmful microorganisms (Bartlett et al. 2002). The major classes of AMPs include small proteins and one or two amino acids.

These peptides are displaying both hydrophilic and hydrophobic surfaces, generally acting by forming pores on microbial membranes or disrupting membrane integrity (Tincu & Taylor 2004). There is evidence that AMPs are widespread in invertebrates (Chisholm & Smith 1992). Especially during larvae development, when shrimp post larvae (PL) are particularly susceptible to infectious diseases, some AMPS such as penaeidin are involved with the ontogeny of the immune system through hemocytes (Muñoz et al. 2003). Figure 1.5 summarizes the main functions of the AMPs in crustaceans.

1.8 Research Rationale and Aims

Despite the efforts to study the shrimp microbiota, little is known about the role of novel feed additives and the effect modulating the gut microbiota and the nonspecific immune response. Thereupon, the aim of this study was to investigate the ability of a commercial immunostimulant, namely the Functional Feed Additive (FFA), to improve growth, performance, and survival of farmed shrimp *Litopeaneus vananmei*, with a more comprehensive analysis in the modulation of the gut microbiota under controlled

experimental conditions. The study evaluated growth performance, feed utilization efficiency and several physiological and health stats in addition to gut integrity and microbial ecology. The main purposes of this study are to identify the effects of different dietary inclusions of β-glucans, Yeast and herbs blend and a tuna hydrolysate in Pacific white shrimp concerning basic parameters such as specific growth rate (SGR), feed conversion ratio (FCR) but mainly growth, survival and analyse the chemical composition, the proximal composition of the organisms, perform a histology and gut analysis, and to identify the biological potential and environmental and microbial ecological aspects relating to the composition of gut microbiota in cultured shrimps and their health status.

1. Our introduction in mainly focus on prevous work with FFA in shrimp nutrition with a strong emphasis in gut integrity, performance and previous scientific work been done in Mexico.
2. Preliminary trials to assess a nutritional trial under commercial conditions with the use of floating cages estimating the influence of a novel feed such as additives on shrimp performance and survivals.
3. Assessing the effect of four types of β-glucans on the immune response and gut health from shrimp benefits in shrimps during a 14-week trial in a commercial farm.
4. To evaluate the modulation of bacterial communities with the inclusion of YAH and some aspect of nonspecific immune response and understand the role of shrimp microbiome when fed with novel feed additives, such as YAH.

5. To evaluate the effect of Tuna Liquid Hydrolysate, at two different inclusion levels, on the growth performance and to evaluate the bacterial community modulation from the posterior intestine of *Litopenaus vannamei.*

CHAPTER 2: General Materials and Methods

2.1 Overview

All of the experimental animals utilized within these studies were obtained from dedicated commercial shrimp hatcheries, *Litopeneaus vannamei* were primarily selected as the experimental specie due to the global importance and the Mexican market. Due to differences in experimental locations and water characteristics, specific protocols for each trial are detailed within respective chapters.

Table 2.1 Summary of conducted experiments

	Chapter 3	Chapter 4	Chapter 5
Species	*L. vannamei*	*L. vannamei*	*L. vannamei*
Initial shrimp weight (g)	3.00 g ± 0.25 g	3.00 g ± 0.25 g	3.00 g ± 0.25 g
Final shrimp weight (g)	24 g	23 g	12 g
Feed profile (CP/F)	35/8 %	30/7 %	25/7 %
FFA evaluated	β-glucans	Yeast & Herbs	Tuna hydrolysate
Inclusion rate (%)	0.2 - 4 %	0.5 - 1 %	1- 2 %
Nutritional trial duration	142 days	102 days	56 days
Analysis	Performance Proximal composition, Histology	Performance, DNA extraction, PCR, HTS- QIIMME	Performance, DNA extraction, PCR, HTS- QIIMME
Salinity (mg. l^{-1})	2 – 3 ppt	33-35 ppt	4-5 ppt
Temperature °C	23 – 31	27 – 34	28 – 33
Season of the year	Spring 2015	Summer 2016	Summer 2017

2.2 Farm site and experimental unit

2.2.1 Experimental desing and farm description

The experiment trials were perform under commercial shrimp farm condition, in where commercial diets were formulated using domestic ingredients, all feed trial were executed in a shrimp ponds by using floating cages, activities occurred in the pond therefore all maintenance, feeding, weighting and evaluating occurred in similar pond conditions, with the aim of analysing the effects of FFA in survival, growth, feed consumption, proximal composition and intestinal morphology and intestinal microbial modulation. The shrimp initial body weight was 3.00 g ± 0.25 g organisms were randomly distributed 100 per cage (Final density per cubic meter is 68.5) and fed on a percentage of body weight ration and tray observation over the course of two times daily. The water parameters were maintained with acceptable values for shrimp culture during the trial period, except when heavy storms hit the farm as seen on the water quality results with drops of temperature and increase in dissolved oxygen.

- Shrimp farm "La Perla del Real" is located 7.5 kilometers from Tecoman Colima (Fig 2.1) and consisted of six earthen pounds of 7,000 m2, with liner and sandy bottoms, aerators, an independent power source, two water wells of 20 liters per second, with 2-3 ppt salinity (chapter 3)

- Experimental unit and shrimp farm "El Tortugario" Cuyutlan, is located 2.2 kilometers from Cuyutlan Colima, a 1,500 m2 pond with high aeration 24 horse power per hectar in where marine water is pumped from an artisanal well by the sea side, sandy bottom with liner, this pond constructed for the course of trials

and further activities in marine conservation. During the experiment, water parameters, plankton, DOB and other parameters weren't compromised, salinity was kept at 33- 35 ppt. (chapter4)

- Shrimp farm "Los Tucanes" is located 22.5 kilometers south of Tecoman, Colima, is an intensive shrimp farm from Azteca Group, with similar conditions in the region, low salinity, 2,500 m2 ponds, paddle wheels, 4-5 ppt salinity. (chapter 5)

Trials were executed in shrimp pond under constant supervision during day and night. Water and soil analysis of the pond were conducted before the trial to validate that the site complied with the experiment requirements (Tables 2.3 and 2.4)

Figure 2.1: Geographic Location from our farm site in Tecoman, Colima, Mexico.

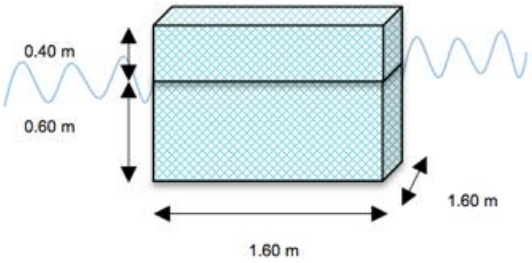
2.2.2 Cages construction and dimensions

In order to evaluate the effect of dietary supplementation with FFA in the Pacific white shrimp under commercial conditions, floating net cages were used for *in vivo trials*

(various biological entities are tested on whole), this technique has been used in other countries as a sampling tool to evaluate different aquatic organisms (Mariojouls, 2008). In the late 1970's A group of French scientists (IFREMAR) were among the first to use cages for experimental purposes. At our site, raw materials were delivered at the farm, 20 tailor-made net floating cages used for all 3 trials, were built with the help from farm technicians and fisherman, based on designs that have been used previously and adjusted to domestic materials available. The cage frame consisted of high quality 2" PVC (polyvinyl chloride) where the pipes were fixed to each other by their ends, the net was 2.5 mm with 1.2 m long and 1.2 m high, a third flexible net was attached at the top to prevent the shrimp from jumping and predators. (birds, frogs, snakes and other)

Table 2.2 List of materials and specifications of the custom – made cages (unit)

Materials per unit cage	PVC	P. Per net	Bird net	Rope	Glue	Notch	Hours labor
Measures	20 m	6.8 m^2	2.25 m^2	35 m	2 oz	600	6-7 h
Specifications	2 ´¨H	¼ ¨net mesh	½ inch	1 mm	Blue marine	Marine	2 people



Floating cages (volume) m^3		
Lenght	1.6	m
Hight	1.65	m
Depth	0.6	m
Volume	1.58	m

Figure 2.2 Floating net cage design of 1.58 m^3

Figure 2.3 A). Experimental arrangement B) Cage frame and net installation, C) Feeding tray and buoyant D) Experimental shrimp pond view.

Below are listed some advantage from the use of floating cages with shrimps as an experimental tool to evaluate shrimp diets under commercial conditions.

- Each replicate is subjected to similar environmental conditions within one pond, removing the pond effect as a factor, whilst maintaining exposure to the same stress factors and conditions that are common in commercial shrimp ponds
- Isolation from the other organisms in the system.
- Better control of feed delivery and recording of feed consumption, as this is applied using PVC trays with the same characteristics.
- Periodic evaluation to know the organism status and survival.
- Detailed control of feed intake per cage, keeping notes on the daily consumption allowing to be adjusting the values as required and noticing the differences between diets accurately.
- A single anchoring system helps to avoid bottom decompose, organic matter builds up and fouling accumulation.
- Requires less than 1500 total organisms to be used in the study
- Requires smaller volumes of feed and personnel to run the experiment

2.3 Experimental animals and housing

All Pacific white shrimp post larvae were obtained from a local shrimp hatchery named Aquagranjas S.P.R located in Tecuanillo, Colima.

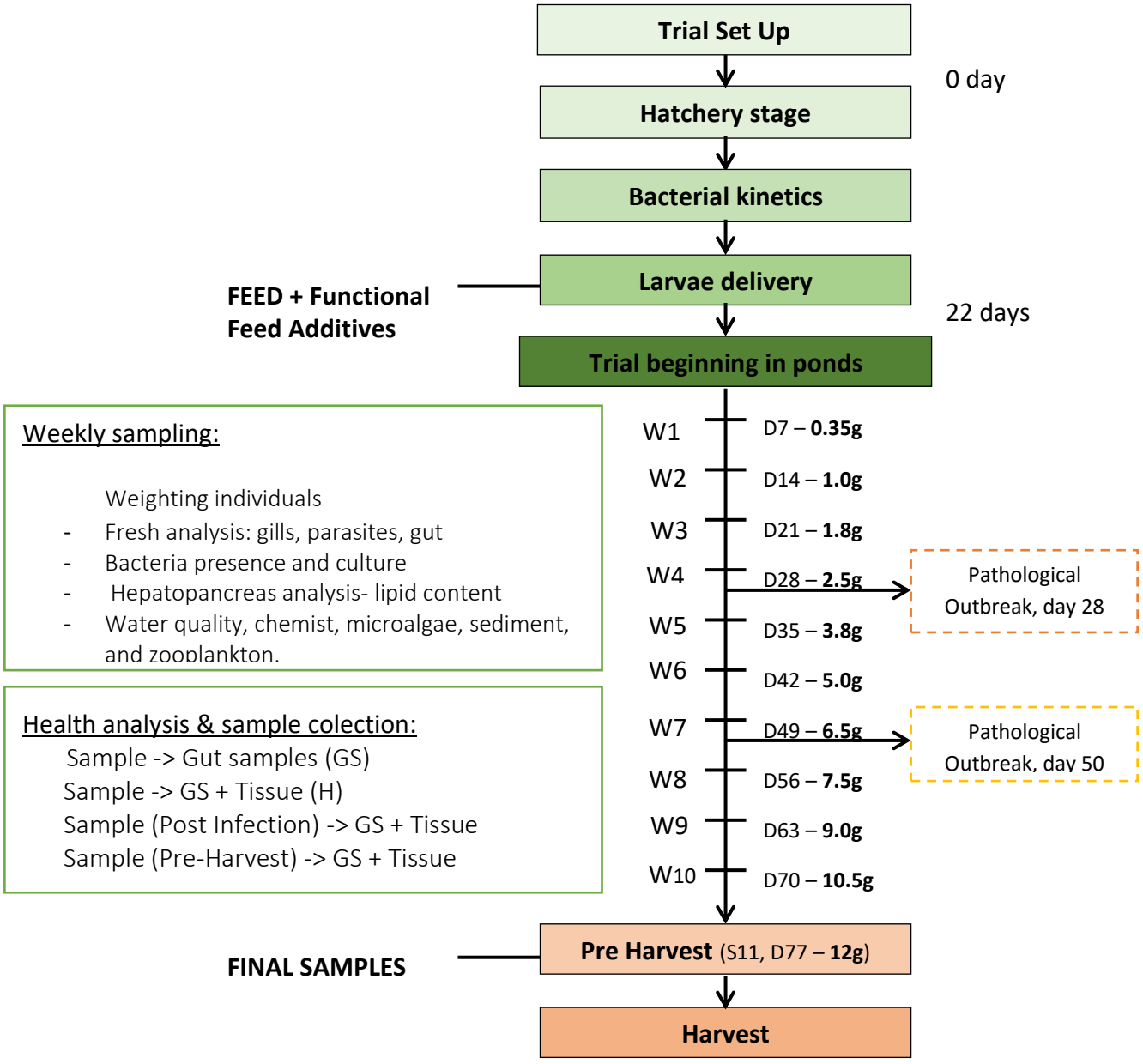
The initial post larvae (PL) size was 20 PL, primarily the PLs were reared with the use of a concrete nursery supported with blowers, polyvinyl chloride (pvc) pipe as diffusion and a 2" inlet water from a 40 m well under a greenhouse. Intense caring and housing occurred for 15 days at a density of 20-18 $pl.l^{-1}$. post larvae per litter and acclimatized from 20 ppt to 7-8 ppt salinity at a range of 1 ppt per day, leaving the last 5 ppt drop to manual acclimatization when transferred from the nursery tanks into the ponds at 400 mg size using 20 liters bucket with oxygen saturated water from nursery.

After 20 days from stoking in the ponds shrimps where collected with 1.5 ml casting nets and were kept in one cage while weighing and counting each individual at 3.00 ± 0.25 g size juveniles. This particular size-stage was selected to start the trial to avoid shrimp from escaping, easy to be selected and the minimum feed size manufacturing at 0.9 – 1.2 mm micro pellet was possible to produce a tailor-made diet with the inclusion of β-glucans in presence, 2.5 tons of feed per treatment.A total of 2000 individual shrimps were place inside cages, within 48 hours during night to avoid heat and light stress, minimal loses where recorder and final density was reach at 100 shrimp per cage, with a final density of 62.5 juveniles per cage cubic meter.

2.4 Pathogenic pathway

This is a self made represent diagram of the infectional pathway at our farm site, this scenario can change accrodign to density, season, temperature and strain been used in the ponds, from our observation during trials with shrimp and coparations with the pond podruction shrimp infection sintoms and historical infection methodology.

Figure 2.4: Feed additive evaluation pathway (W: weeks; D: days)



Current trials with functional feed additives can deliver better results in terms of survival and performance with a chronological plan or analogy is adapted. This include the product strategy according to specific shrimp health requirement, stoking density, season and management to avoid mass mortalities in early stages due to bacterial infection (*Vibrionaceae spp)* water quality changes and biomass increase (Figure 2.4)

2.5 Water quality and experimental conditions

Temperature, pH and dissolved oxygen were measured two times per day using a pH Meter (Phep® by Hanna) and DO meter (YSI 55 model) and ammonia levels were monitored in weekly by the NitriVer® 3 diazotization method. During the whole experiment, salinity was maintained from 2 to 35 ppm depending on the site, water temperature was constant during most of the experiment with an average of 29℃ and some fluctuations when climate was challenging. The dissolved oxygen was fluctuant during trial with an average range from 2-12 ppt, this high variability was caused by a number of the situations when performing long production cycles.

2.5.1 Farm water conditions

Water quality and earth mineral profile is essential for shrimp farming in low salinity sites, multiple ions and specific combinantion of minerals are needed for methabolic funtions and osmoregulation.

Table 2.3. Farm "Acuicola Los Tucanes" located in Tecoman, site soil characteristics

Parameter	Result	Unit	Instrument (Hydrometer)
Texture			
Sand	51.38	%	Bouyoucos
Clay	1.62	%	Bouyoucos
Silt	47	%	Bouyoucos
Textural classification	Sandy Loam	Sandy Loam	Texture triangle

University of Guadalajara, Soil Department.

Table 2.4 Farm water mineral profile.

Parameter	Result	Unit
Salinity	2-3	$mg.l^{-1}$
pH	8.1 - 9	H+
$CaCO^{+}$	170/220	$mg.l^{-1}$
Electro conductivity	+3000	$dS.m^{-1}$
Mg	0.05	$mg.l^{-1}$

The turbidity of the pond number 3 remained in good levels with abundant phytoplankton during the trial period; pH was within acceptable ranges (8.2 - 9.0) and total ammonia content remained within a range of 0.0 – 0.01 $mg.l^{-1}$. Biomass calculation was performed every two weeks with the aim of monitoring the growth rate of shrimp over time, mortalities and feed consumption, a sample of 40 animals per cage were weighted. After the experimental period, shrimp from each cage were counted and weighed to determine the survival rate, final body weight, survival and density using a digital scale (Ohaus, Model 1020).

During the experiment with Betaglucans, water temperature was constant during most of the experiment with an average of 28℃ although some storms occurred in the course of the trials with the exception of a storm, which happened between tropical storm in which the temperature dropped to 23°C, this was reflected in an increase in the concentration of DO in the pond (up to 8 ppt).The DO remained stable during trials with an average range of 3-5 ppt with some fluctuations caused by the normal effects of cultivation, which are directly related to the biomass in the pond.

2.6 Experimental diets and formulation

All experimental diets were formulated with different protein profiles, based on a commercial formula previously used in each region, a rich inclusion of regional protein sources, cereals and fats were implemented to meet shrimp farming requirements in low salinity systems.

First control treatment was a thirty five percent crude protein eight percent fat for the β-glucans, second experimental diet was formulated for the yeast and herb experiment but was not mixed with the YAH, instead the YAH was added on top with vegetable oil, lastly the third experimental diet was a slim formulation with only twenty five percent crude protein in where the TLH was added in the mixture, all formulas to be iso-nitrogenous and iso-lipidic, using conventional ingredients, to meet the known nutritional requirements of shrimp (NRC 2011).

2.7 Experimental Feed Manufacturing

2.7.1 Nutrimentos Abafor S.A de C.V

For our first trial with betaglucans worked with a domestic company Abafor (http://www.abafor.com/contacto.html), were feed additives were added to the mixture, and the dough was pelletized using a 2mm diameter die using a California Pellet 1000 of 35 horsepower. The mixture was compacted into pellets and steamed at a central temperature of 88°C. At the final phase of the powder production, the pellets passed through a dryer resulting in 1.5 - 2 mm pellets.

2.7.2 Nutrimentos Acuicolas Azteca SA de CV

For our second and third experiments shrimp diets were tested in collaboration of Azteca R&D team (http://aztecamexico.mx/contacto/), Mexican company located in San Pedro Tlaquepaque city. For our TLH dietary experiment, feed was commercially manufactured using a 250-HP California Pellet Mill (CPM) and producing batches of two to five metric tons per hour at 2 mm size pellet.

2.8 Growth performance and feed utilization.

Feed adjustment, growth, and health characteristics were recorded and observed every week by weighing a pooled sample of the population (n= 40 PL per cage, i.e., 30% of the population per cage). This monitoring allowed the calculation of growth performance, zootechnical parameters, FCR (feed conversion ratio), and an inferred economic analysis.

The Feed Conversion Ratio (FCR) was calculated using Equation 1. Specific Growth Rate (SGR) was calculated using Equation 2.

$$FCR = F/G$$

FCR= Feed Conversion Ratio
F = weight of feed provided
G= weight gain

(Eq 1)

$$SGR = \left(\frac{(\ln \text{final shrimp weight} - \ln \text{initial shrimp weight})}{\text{period of time}}\right) \times 100$$

(Eq 2)

$$\text{Survival (\%)} = \frac{(\text{intital count of shrimp in the cage} - \text{final no. of shrimp in the cage})}{\text{initial count of shrimp in the cage}} \times 100$$

(Eq 3)

The feed regime used in the experiment was according to the farm feeding table (Table 2.5), two times per day is the regular rate after nursery stage when juveniles are transferred into the ponds, during the weeks under trial feeding ratio was adjusted individually by checking the trays every day before feeding (Nunes, 1996). The feed adjustment was important because the shrimp behave differently from one cage to the other, passing through distinct stages of growth and molting.

Feed adjustment, growth, and health characteristics were recorded and observed every week by weighing a pooled sample of the population (n= 40 PL per cage, i.e., 30% of the population per cage, see annex 7.8) This monitoring allowed the calculation of growth performance, zoo technical parameters, FCR (feed conversion ratio), and an inferred economic analysis.

2.9 Sample collection

Biomass sampling was performed every two weeks on the first trial with betaglucans and the following trial I was weighting every week, with the aim of monitoring the growth rate of shrimp over time and to increase cycle length, there by mimicking the commercial conditions, mortalities and feed consumption a sample of 40 animals per cage were individually weighted, all weighting was performed with accuracy of 0.1 g (Ohaus, Model 1020), in tared plates. After the experimental period shrimp in each cage were individually counted and weighed, mortalities were recorded along the trial to determine the final body weight, survival and final biomass increase.

For the intestinal morphology analysis, five shrimp from each replicate were sampled and prepared for histological analysis. Samples from digestive organs containing tissue were preserved for analysis using a 10 % formalin Davison fixative solution, the application was performed using a 5 ml syringe injecting the fixative (Lighter et al., 1996) in specific parts of the shrimp as follows: hepatopancreas (head), anterior section, mid-section and posterior section (abdominal section number 6) after 48 hours the shrimp were transferred into pure ethanol molecular grade (Sigma).

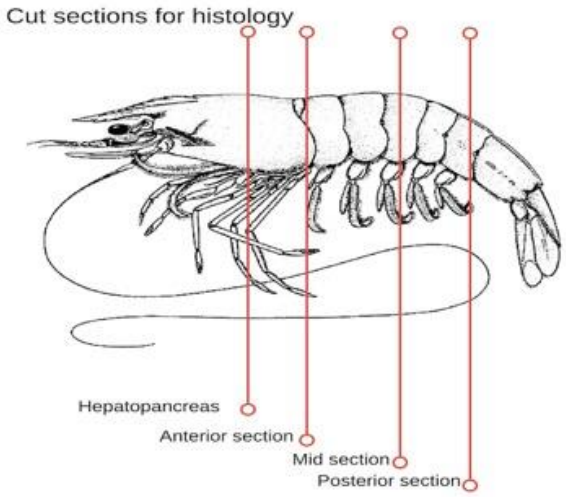


Figure 2.5 Shrimp sections and abdominal body segments

Sample collection for bacterial DNA. A total of 84 shrimp in each trial were randomly sampled, i.e., 28 shrimp per treatment and 7 per cage. Animals were anesthetized with hypothermia using ice water at 14 ◦C, and shrimp were submerged and kept in isolated containers for analysis. Animals were euthanized by thermal shock (33 °C to 9 °C), carcass surfaces were washed with 70% (v/v) ethanol and shrimp samples from the posterior gut with its content were collected, using sterilized tweezers and scissors. Samples were immediately fixed in 70% (v/v) molecular grade ethanol, stored in sterile 2 ml microtube, and kept under -20 ℃ for subsequent analysis. A total of 48 shrimp were randomly sampled, 4 individuals per cage a total of 16 shrimp per treatment. Samples were transported under cold conditions to the University of Plymouth (UK), with the appropriate Home Office importation Licence #TARP/2015/2019.

2.10 Euthanasia

Animals were euthanised by thermal shock (From ~ 31 °C to 9 °C) once the organisms were euthanized under thermal, a 500 ml container with a fixative solution was used to contain the shrimp samples; after 48 hours, the shrimp were transferred into 100% ethanol for H&E analysis and DNA samples for microbial analysis were kept in Eppendorf of 1 ml with molecular ethanol at -20 ℃ for further analysis.

2.10 Proximal analysis from shrimp

At the end of the experiment, 35 samples per treatment from each experiment were dried to a constant weight using an auto oven (Genlab Oven, digital 40 liters, 40-230 ◦C) at a temperature of 103 ℃, with the support from Consorcio Super S.A. de C.V. feed analysis laboratory, all samples were kept in dry sealed plastic bags before been shipped to the University of Plymouth, UK.

2.11.1 Moisture

The moisture content was determined by subtracting the dry weight of the sample from the wet weight.

$$\text{Moisture (\%)} = \left(\frac{(\text{wet weight (g)} - \text{dry weight (g)})}{\text{wet weight (g)}} \right) \text{x } 100$$

Equation 2.4. Moisture (%). (Eq 4)

The dry matter (DM) was calculated with the following equation:

$$\% \text{ Dry matter} = 100 - \% \text{ moisture} \quad \text{(Eq 5)}$$

Equation 2.5 Dry matter (%).

2.11.2 Crude protein

To determine the protein content, the nitrogen content was measured using AOAC, 1995. The sample was subjected to a digestion and distillation process to finally quantify the nitrogen content by titration using a concentrated sulfuric acid.

After obtaining the nitrogen content of the sample, the following equation was used to calculate the protein content. (Kjeldahl Method)

$$\text{Protein } (\%) = \frac{(\text{Nitrogen } (\%) * 6.25)}{\text{Efficiency factor}} \quad \text{(Eq 6)}$$

Equation 2.6. Protein (%)

2.11.3 Fat

To determine the content of lipid in the sample the Soxhlet method was used (Soxhlet et al. 1897) The lipids in the samples were extracted using petroleum ether in the Soxtherm Extraction Unit. (Fisher, scientific)

$$\text{Fat}(\%) = \frac{\text{Lipid weight (g)}}{\text{Initial weight of sample (g)}} \text{ x } 100 \quad \text{(Eq 7)}$$

Equation 2.7. Fat (%).

2.11.4 Ash

The ash content in the samples was determined using the direct method which is an adaptation of AOAC Official Method 923.03, 1995. The ash content was determined by incinerating the sample at a temperature of 550℃ in a muffle furnace for 8 hours.

$$\text{Ash} = \left(\frac{\text{ash weight [g]}}{\text{sample weight [g]}}\right) \text{ x } 100 \quad \text{(Eq 8)}$$

Equation 2.8 Ash (%)

2.11. 5 Energy

The energy content in the sample was measured using a bomb calorimeter (6200, Parr). One gram of sample was weighed into a crucible and placed inside the decomposition vessel filled with 30 bar of oxygen. Then 2,000 g of water were added to prepare the calorimeter water jacket. The water jacket allowed measuring the heat created by the combustion process as this was transferred to the water jacket and then converted by the internal microprocessor of the bomb calorimeter into the energy value of the sample tested.

2.11 Peptide profile analysis

With the use of analytical methods of exclusion as Chromatography, we analyzed the peptide molecular weight profile of the experimental shrimp feeds with the inclusion of TLH. This method provides a “fingerprint” of the feed ingredients (Lian; Lee; Park, 2005; Stranska-Zachariasova et al., 2016), being an extensive analytical technique, which outcome can result in several benefits, such as product development, quality control, competition analysis, marine raw materials analysis, and aquafeed analysis (Altunok et al., 2016; Habibi et al., 2017; Nolvachai; Kulsing; Marriott, 2017).

Resin column (200- 15000 Da) was used to obtain molecular weight classes, peptides and protein, calibration curve with 7 standard peptides, reading absorbance at 214 nm, % of peptides calculated by interacting area under the HPLC curve.

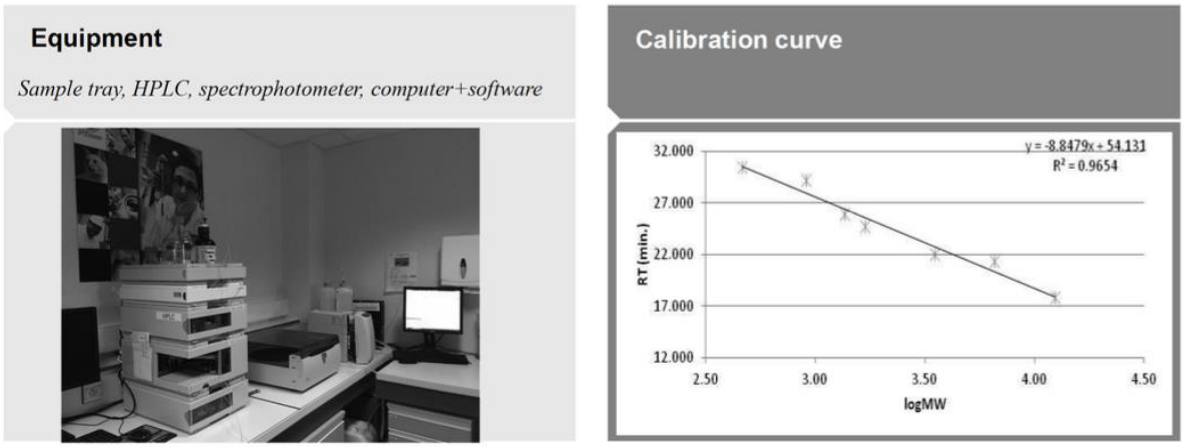


Figure 2.6: HPLC, (Agilent 1290 Infinity series) and calibration curve for establishment the molecular weight distribution profile of peptides

2.13 Histology analysis

All sectioning of species was conducted at 5 Mm thickness using Leica RM2235 microtome (Leica; Buck, UK), with blocks being chilled on a cooling plate (Leica EG1150 H: Bucks, UK) prior to sectioning. Sections were mounted on glass slides and dried at 30°C for 48h.

All intestinal specimens were stained using a Leica Autostainer XL (Leica: Buck, UK). This process was initiated by rehydration in graded ethanol concentrations; twice at 100% followed by 90, 70, 50% ethanol and rinse with distilled water. Stains implemented were haematoxylin and eosin (H&E), and periodic Acid-Schiff with Alcian blue (PAS), cover slips were mounted with DPX and left to dry at 30°C.

Micrographs were captured with Leica DMIRB microscope and Olympus E410 digital SLR camera, at varying magnifications.

2.14 Molecular microbial and DNA extraction

2.14.1 DNA extraction

During the course of this analysis, multiple protocols were evaluated, and I and some of my colleagues from University of Plymouth found inconsistent methods to extract DNA, especially for marine organisms, therefore with the support of other Ph.D. students specially Cecilia De Souza, we develop a tailor protocol to extract bacterial DNA from shrimp intestine. DNA was extracted with the commercial kit QiAmp DNA stoll mini Kit, Qiagen®. Initial steps for sample preparing and lysozyme for bacterial lysis were performed. for more details see (Appendix 7.1)

2.15 High Throughput Sequencing

Molecular biology techniques described in this section were performed at the Microbiology laboratory at the University of Plymouth, UK. DNA extraction was performed shrimp gut using the QIAamp Stool Mini Kit (Qiagen®) and following manufacturer's instructions. In order to enhance the lysis of Gram-positive bacteria, an initial incubation with 50 $mg.ml^{-1}$ of lysozyme for 30 min at 37 ^{o}C was added to the protocol. Extracted DNA purity and quantity were measured using a UV spectrophotometer (NanoDrop™ 2000 Spectrophotometer, ThermoFischer Scientific®), based on the ratio of absorbance at 260/280 nm and 260/230 nm.

A fragment of 350 bp from the hypervariable V1-V2 regions, from bacterial 16S ribosomal RNA (16S rRNA), was amplified through a *touchdown*-polymerase chain reaction (PCR) assay, using the primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and a pool of primers 338R-I (5'GCW GCC TCC CGT AGG AGT 3') and 338R-II (5' GCW GCC ACC CGT AGG TCT 3') (Gajardo et al., 2016). For the *touchdown*-PCR, 1 µl of the DNA template (1 ng/µl) was added to the PCR mix solution containing 25 µl of MyTaq™ Red Mix (Bioline®), 1 µl of each primer (25 pM), and ultrapure DNase free water for a final volume of 50 µl. The amplification cycling profile is presented in Table 3.3. To demonstrate an accurate PCR performance, positive (*Escherichia coli* DNA) and negative (ultrapure water) controls were used in each amplification reaction. Subsequently, the amplified products were analysed by 1.5% agarose gel electrophoresis, with SYBR Safe DNA gel stain (ThermoFischer Scientific®), in TAE buffer at constant voltage (80 V) for ~ 40 min and visualized under UV light.

Pooled PCR products were purified using AMPure XP (Beckman Coulter®), based on the magnetic bead's technology. Finally, purified PCR products were sent to Systems Biology Centre of University of Plymouth UK, Genomics Facilities, for the High Throughput Sequencing (HTS), utilizing Life Technologies Ion Torrent™ Personal Genome Machine™ System (ThermoScientific®).

Table 2.5 Cycling profile of the *touchdown*-PCR amplification

Phase	Temperature (°C)	Time	Cycles
Initial denaturation	94°	7’	
Denaturation	94°	30”	
Hybridization	63° - 53°	30”	10 x touchdown
Elongation	72°	30”	
Denaturation	94°	30”	
Hybridization	53°	30”	25 x
Elongation	72°	30”	
Final Elongation	72°	10’	
Finalization	10°	Until end	

2.16 Bioinformatics QIIME

Raw sequence data were trimmed using FASTX-Toolkit (Hannon Lab), and sequences with low-quality scores (Q < 20) were filtered out. Data were then assessed using Quantitative Insights Into Microbial Ecology (Qiime 1.8.0) (Gajardo et al., 2016). Sequences were analysed using QIIME 1.8.0, and Operational Taxonomic Units (OTUs) were sorted and filtered with 97% of sequence identity. Ribosomal Database Project (RDP) tool was used to assign taxonomic affiliation, with 0.8 of confidence. Alpha and *β* diversity were calculated with ape, vegan, and R. Bacterial richness and diversity were determined with indexes such as Chao1, Observed Species and Phylogenetic diversity. Good´s coverage was also identified. Additionally, Weighted and Unweighted UniFrac

distances were used to estimate similarity and dissimilarity and confirmed with Principal Component Analysis (PCoA). The analysis transforms observed correlated variables into spatial dimension, thus emphazising the variation and correlation among variables, plotting them on an X-axis and Y-axis graph. The taxonomic analysis was estimated with relative abundance graphs at phylum and genus level. LEfSe (Linear discriminant analysis effect size) tool was used to determine differentially abundant taxa between treatments, and significantly different taxa were used to calculate LDA effect size (Segata et al., 2012), with a significant P value < 0.05 and effect size threshold of 2. Finally, the Venn diagram was built to identify the core microbiota, as well as unique and shared OTUs between treatments, using Venny 2.1 software (http://bioinfogp.cnb.csic.es/tools/venny/, Oliveros 2007-2015). Data are presented as mean ± SD. The p-value < 0.05 was considered statistically significant.

2.17 Statistical analysis

For all data, means ± standard deviation (SD) are presented. Statistical analyses were carried out using Minitab version 16 (Minitab® Ltd, Coventry, UK) and IBM SPSS Statistics Base. Data were tested for normality and one-way ANOVA with Turkey´s *post hoc* test was carried out thereafter. If data were not normally distributed, Kruskal-Wallis test was carried out. In all cases, significance was accepted at $p < 0.05$ or $p < 0.01$.

CHAPTER 3: Effects of dietary supplementation with β-glucan in Pacific white shrimp

3.1 Introduction

For the last decade, several studies have been developed about the implementation of natural additives to boost the immune system of shrimps in aquaculture using natural products such as ginger, garlic, yeast, herbs, and β-glucans (Emeka et al., 2014)

Yeast is unicellular eukaryotic microorganisms that are taxonomically placed within the phyla Ascomycota and Basidiomycota within the Fungi Kingdom. They are widely distributed in several natural environments such as soil, freshwater, and seawater. Their number and species distributions are dependent on the concentrations and types of available organic materials. It has been proven that several yeast compounds have significant biological value as reagents, cell proteins, vitamins, pigments and enzymes (Navarrete et al., 2014).

Yeast glucans (β 1-3 and β1-6 linked glucan) and β-1,3 glucan (VST) is derived from cell walls of baker´s yeast like *Saccharomyces cerevisiae* and *Schizophyllum commune*, respectively (Barman et al., 2013). As shown in Figure 3.1, the yeast cell wall is composed of complex polymers of β-glucan (1,3) and (1,6), mannan-oligosaccharide (MOS) and chitin. MOS is located on the surface of the cell wall. Meanwhile, yeast glucans are often present in the inner wall layer and are associated with other cell components such as chitin (Bai et al 2014).

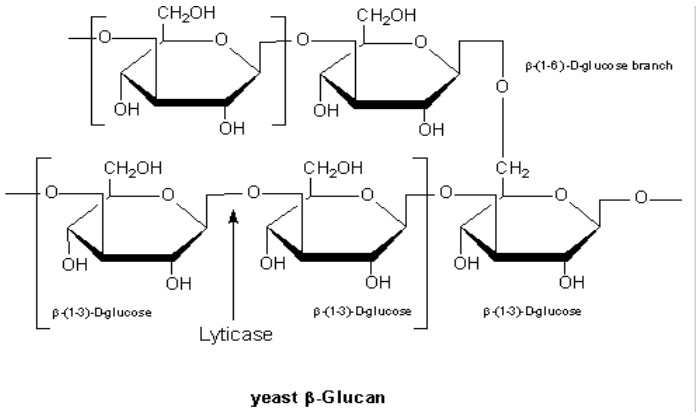


Figure 3.1 Yeast β-glucans structure (Rahar et al. 2011)

The use of β-glucan on shrimp feeds has been around the industry for at least twenty years and the enhancement of vibrio resistance by supplementations of diets (Cruz, 1999) is well documented, the level of inclusion can range from 0.2 to 0.1 per ton of feed is the regular inclusion rate in commercial shrimp feeds.

3.2 Aims and objectives

The main purposes of this study were to identify the effects of different dietary inclusions of β-glucans in Pacific white shrimp concerning basic parameters such as specific growth rate (SGR), feed conversion ratio (FCR) but mainly growth, survival and analyze the chemical composition, the proximal composition of the organisms, perform a histology gut analyzes, among other immunological parameters.

3.2.1 Objectives:

1. To perform a successful In-*vivo* trial with the use of floating cages and tailor-made feeds during a full productive cycle in our farm site.

2. To estimate the influence of multiple β-glucans blends in shrimp performance and survival.

3. To analyze the proximal composition from shrimps and the gut morphology an intestinal change on the posterior part.

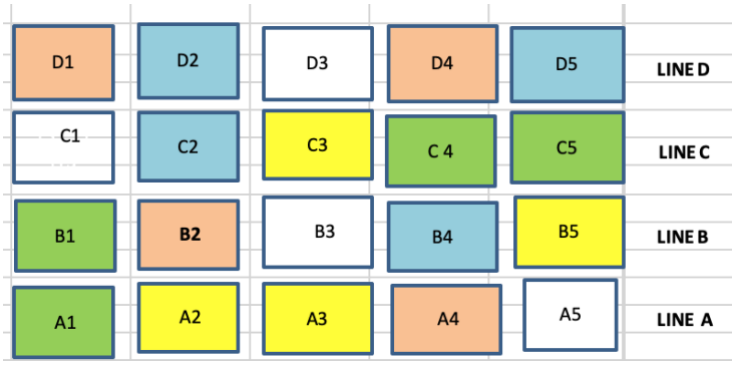
4. To analyze the economics from the use of β-glucans in shrimp diets under commercial conditions.

3.3 Materials and Methods

3.3.1. Experimental unit and site

An experimental area of 2000 m^2 was located for the trials with floating cages attached to a double anchor point, which consisted of five treatments with four replicate net cages placed randomly inside the pond with the objective of achieving the same environmental conditions for all cages (Fig 3.2).

The farm consisted of six earthen pounds of 0.7 hectares each one with liner and sandy bottoms, aerators, an independent power source, two water wells of 20 liters per second, accommodation, raceways. All Pacific white shrimp postlarvae were obtained from a local shrimp hatchery named Aquagranjas S.P.R located in Tecuanillo, Colima.



Cage Number	Treatment	Color ID
C1,D3, B3, A5	Control	White
C2,D2, B4, D5	Beta S	Blue
A4,D1,B2, A4	Beta S- Biolex	Red
A1,B1,C4,C5	Excel	Green
A2,A3,B5,C3	3 components	Yellow

Figure 3.2 Experimental arrangement and cages ID.

A total of 2000 individual shrimps were place inside cages, within 48 hours during night to avoid heat and light stress, minimal loses where recorder and shrimp were stocked at 100 shrimp per cage, with a final density of 62.5 juveniles per cage cubic meter as mentioned in chapter 2.

3.3.2 Diets supplemented with β-glucans

A high-quality shrimp diet was formulated, based on a previous formulations used previously in this region, a rich inclusion of premium quality protein sources to meet high-density farming requirements in low salinity systems were used on this trial to asses zootechnical performance and eventually decrease the quality of ingredients and the crude protein (Table 3.1), a control treatment and a basal formula with the addition of β-glucans from Leiber GmbH, Germany a brewers yeast source.

Diets were formulated to be iso-nitrogenous and iso-lipidic, to meet the known nutritional requirements of shrimp (NRC 2011). For each treatment, the additives were added to the mixture, and the dough was pelletized using a 2mm diameter die using a California Pellet 1000 of 35 HP (horsepower) in where the mixture was pelletized and steamed at a central temperature of 88°C, final phase of the production in when the pellets passed through a dryer at 95 °C resulting in 2 mm size semi dried feed.

Table 3.1 Feed formulations, nutrition profile and characteristics of each diet containing β-glucans, proximate composition as basal diet.

Ingredients (g kg^{-1} diet)	Control	*B*-S	*B*-S Plus	ExCel	3-Component
Fish meal [1]	17	17	17	17	17
Soybean meal [2]	21	21	21	21	21
Poultry meal [3]	18	18	18	18	18
Wheat flour [4]	36	36	35	35	35
Lecithin [6]	1	1	1	1	1
Fish oil [7]	3	3	3	3	3
Soybean oil [8]	1	1	1	1	1
Vitamin premix b [10]	1	1	1	1	1
Mineral premix c [11]	1	1	1	1	1
Vitamin C [12]	0.3	0.3	0.3	0.3	0.3
Choline chloride (50%) [13]					
Antifungal [14]	0.1	0.1	0.1	0.1	0.1
B-glucan content (g kg^{-1} diet) [9]					
B S	0	0.20	0	0	0
B-S Plus	0	0	0.20	0	0.20
Biolex MB40	0	0	0.80	0	0.80
ExCel	0	0	0	3.00	3.00
Analysis (g kg^{-1} diet; dry weight basis)					
Moisture (%)	6	6	6	6	6
Crude protein (%)	35	35	35	35	35
Crude lipid (%)	8	8	8	8	8
Ash (%)					

[1] 58/7 Tuna byproduct fishmeal, Mexico
[2] 55/3 Soybean meal from USA
[3] 62/6 Poultry meal, USA
[4] 7/2Wheat flour, Mexico
[6] Lecithin from Mexico
[7] Fish oil, Guaymas, Mexico.
[8] Soybean oils, USA
9 β-glucans
[10] Vitamin premix b, VIMIFOS, Mexico
[11] Vitamin premix c, VIMIFOS, Mexico
[12] Vitamin C, Stacey VIMIFOS, Mexico
[13] Choline chloride (50 %), VIMIFOS, Mexico
[14] Antifungal, VIMIFOS, Mexico

3.3.3. Feed management and strategy

The feed regime used in the experiment was according to the farm feeding table (Appendix 7.8) at 5% to 3 % body weight, two times per day is the regular rate after nursery stage when juveniles are transferred into the ponds, during the 35 weeks the feeding trial the feeding ratio was adjusted individually by checking the trays for uneaten feed every day before feeding (Nunes, 1996). The feed adjustment was important because the shrimp behave differently from one cage to the other, passing through distinct stages of growth and molting.

3.3.4 Water quality monitoring

Temperature, pH and dissolved oxygen (DO) were measured two times a day and ammonia levels were monitored on a weekly basics. During the whole experiment, salinity was maintained at 2 ppm, ambient water temperature was constant during most of the experiment with an average of 29°C and some fluctuations when weather was challenging the dissolved oxygen was fluctuant during trial with an average range from 2-12ppt, this high variability was caused by a number of the situations when performing long production cycles.

The turbidity (microalgae concentration) of the pond remained in good levels (20-30 cm secci) with abundant phytoplankton during the trial period; pH was within acceptable ranges (8.2 - 9.0) for pysiological maintenance and total ammonia content remained within a range of 0.0 – 0.01 $mg.l^{-1}$.

After the period of 143 days, shrimp in each cage were counted and weighed to determine the survival rate, final body weight, survival.

During the experiment some storms occurred, where temperature dropped to 23°C. This was reflected in an increase in the concentration of DO in the pond (up to 8 ppt) (Figures 3.3 and 3.4). The DO remained stable during the remaining of the trial with an average range of 3-5 ppt with some fluctuations caused by the normal effects of cultivation, which are directly related to the biomass in the pond.

Table 3.2 Water quality parameters in experimental ponds.

Parameter	Results
Water temperature range	28 - 32 °C
pH	8.2 -9.0
Dissolved Oxygen (DO)	2.5 – 6.0 ppt
Total ammonia contents	0.0 – 0.01 mg. l^{-1}

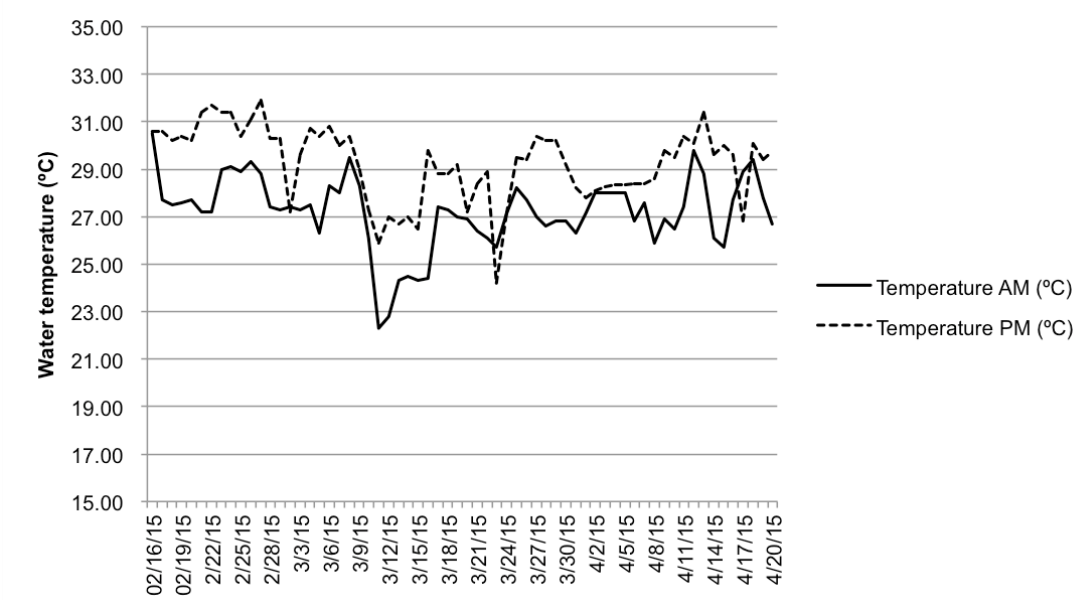


Figure 3.3 Water temperature during the experiment period.

(16th of feb 2015 to 20th april 2015)

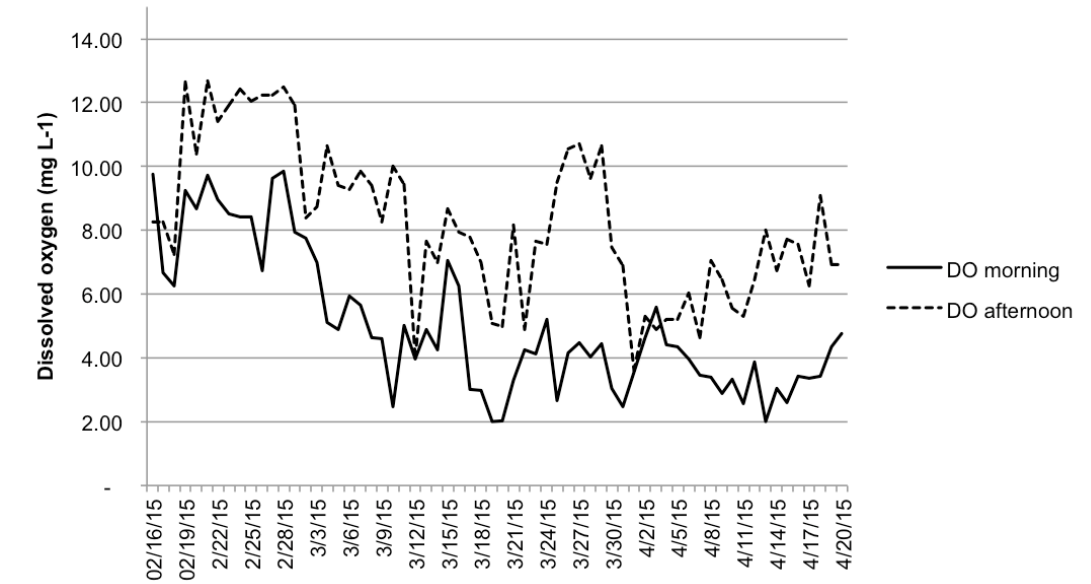


Figure 3.4 Dissolved oxygen during the experiment period.

3.3.5 H&E staining

With the objective to analyze the effect of β-glucan on intestinal morphology, five shrimp from each replicate were sampled and prepared for histological analysis. Samples from hepatopancreas, mid gut, posterior gut digestive organs containing tissue were preserved for analysis using a 10% formalin fixative solution, the application was performed using a 5 ml syringe injecting the fixative. After 48 hours, the shrimp were transferred into 100% ethanol. All samples were taken at the site and the data was collected during the harvest of the cages; the shrimp was weighted before dehydration occurred.

Samples were removed from the fixative solution and specific slides were taken from five shrimp from each treatment and were selected and prepared for H&E histology analysis in where posterior gut was the main focus.

3.3.6 Proximate analysis from shrimp

At the end of the experiment, a total of 35 samples per treatment were dried until a constant weight was achieved using an auto oven (Genlab Oven, digital 40 liters, 40-230 °C) at a temperature of 103 °C. The samples were kept in dry sealed plastic bags properly labeled before been shipped to the University of Plymouth, UK. Some of the parameter analyzed were by calculation, energy, protein, lipids, moisture, and ash.

3.3.7 Statistical analysis

For all data, means ± standard deviation (SD) are presented. Statistical analyses were carried out using Minitab® Ltd, Coventry, UK) and IBM SPSS Statistics Base. Data were tested for normality and one-way ANOVA with Turkey´s *post hoc* test was carried out thereafter. If data were not normally distributed, Kruskal-Wallis test was carried out. In all cases, significance was accepted at $p < 0.05$ or $p < 0.01$.

3.4 Results

3.4.1 Gross observations

During the 35 weeks feeding trial, the weight gain in the cages was below by nearly 50% in comparison with the shrimp from the pond, due to a number of reasons in where I assumed density played a key role. Between treatments shrimp final weight was also found similar between treatments with an average of (18.9±1 g)

When analyzing survival, we found significant better survival ($p<0.05$), compared to control diet, in percentage, 43.5, 56, 66.2, 65.5 and 71.5 treatments, respectively.

Specific growth rate (SGR) also showed significant differences between treatments ($p<0.05$) compare to control diet and most cases diets with β-glucans performed better, and lastly the biomass expressed in $kg.m^3$ in between cages was significant higher with treatments groups fed with β-glucans suggesting better resistant and tolerance to stress conditions.

In Figure 3.5, it is possible to notice that the diets that had better survival were *B*-S Plus, Excel and 3 Component, compared to the control group. Nevertheless, according to Figure 3.6, the diets that presented the best performance, in weight gain terms, were the control group and the ExCel, after 35 weeks, despite no statistical difference, taking into account that the density is correlated to the weight gain in culture conditions.

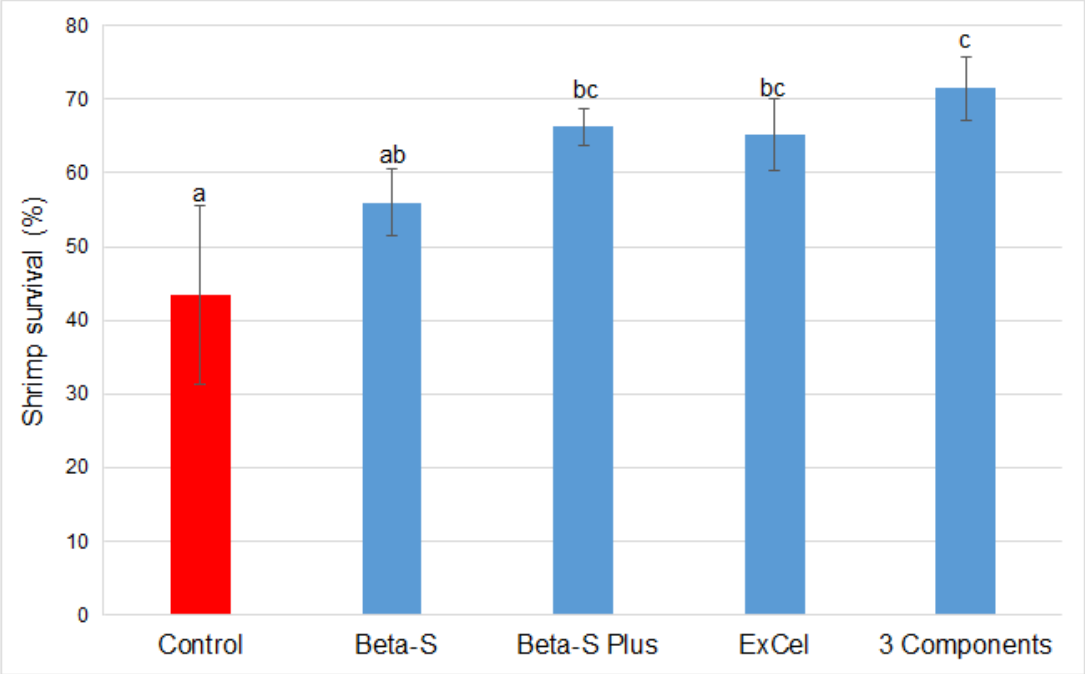


Figure 3.5: Survival of *Litopenaeus vannamei* with four yeast derived β-glucan products and a control diet. Data are average and were calculated at the end of the trial after 143 days. Different letters mean significant differences (ANOVA + Tukey, p=0.0002).

As consequence of the increment on the survival rates in *B*-S Plus, Excel, and 3 Component treatments, the shrimp density in the floating cages increased, generating a greater biomass presence and slowing the growth rates; this observation can be

attributed to the fact that these three treatments tended to have lower weight gain between all, especially *B*-S Plus and 3 Component. In figure 3.6 shows the final average weight, and in figure 3.7 is possible to appreciate the direct relation between survival and density.

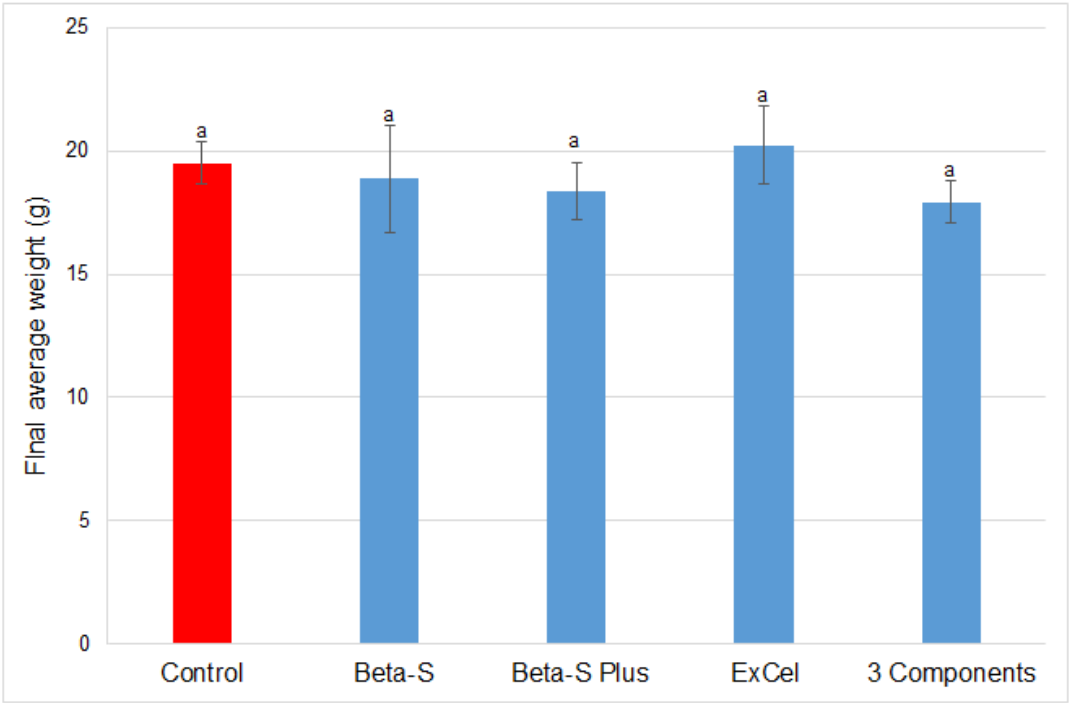


Figure 3.6: *Litopenaeus vannamei* final body weight in reference to four yeast derived β-glucan products and a control diet. Data are average and shrimp were weighed individually at the end of the trial, after 143 days. Similar letter means no significant differences (ANOVA + Tukey, p=0.2056).

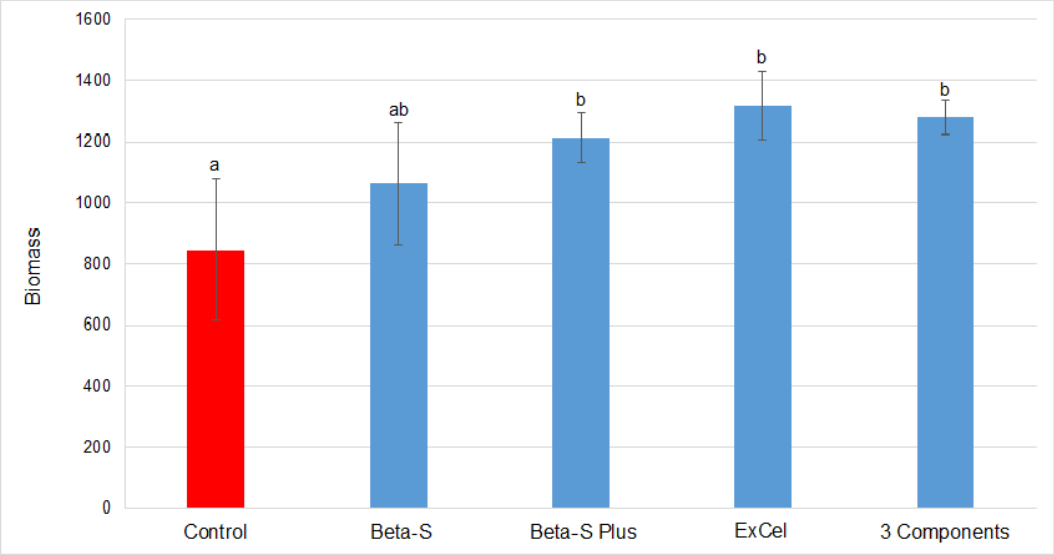


Figure 3.7. Biomass between treatments (grams/ cage). Net biomass was calculated in 1.56 m^3 flowing cages. Data are averages with significant differences (ANOVA + Turkey, $p < 0.0031$).

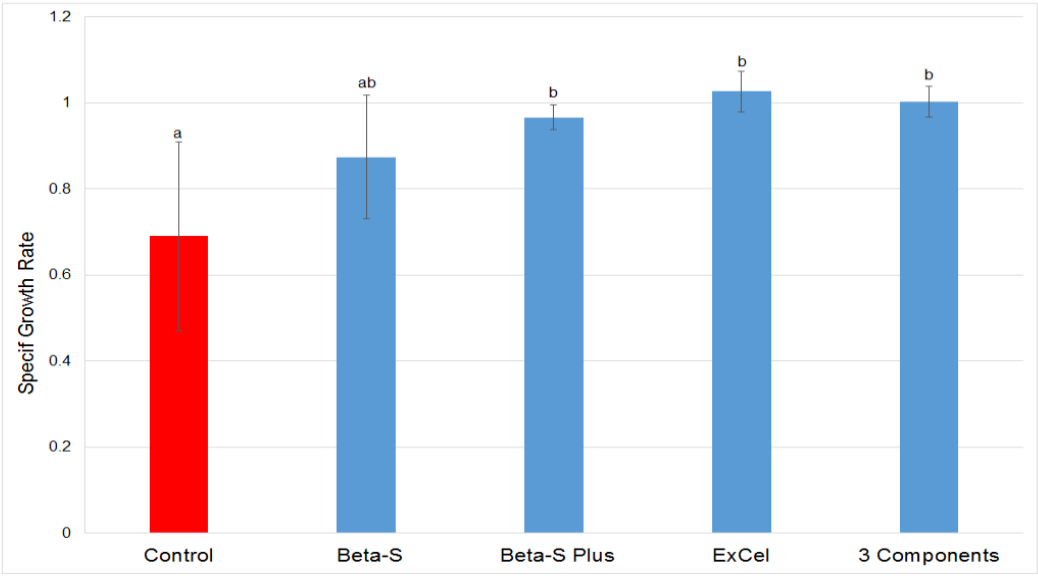


Figure 3.8: Specific Growth Rate (SGR) of *Litopenaeus vannamei* related to four yeast derived β-glucan products and a control diet. SGR was estimated based on initial and final shrimp weight, after 143 days. Data are average. Different letters mean significant differences (ANOVA + Tukey, p=0.0081).

Regarding the specific growth rate, calculated based on final and initial shrimp weight, Houssain (2008) reported the rate of SGR for shrimp is 1.2 - 0.9%. Thus, we can see that, in general, all treatments have a value within this range. Moreover, animals that received *B*-S Plus, ExCel and 3 Components presented a better SGR than the control group (Fig. 3.8), significant differences between treatments ($p<0.05$) compare to control diet in all cases diets with *β* glucans performed better.

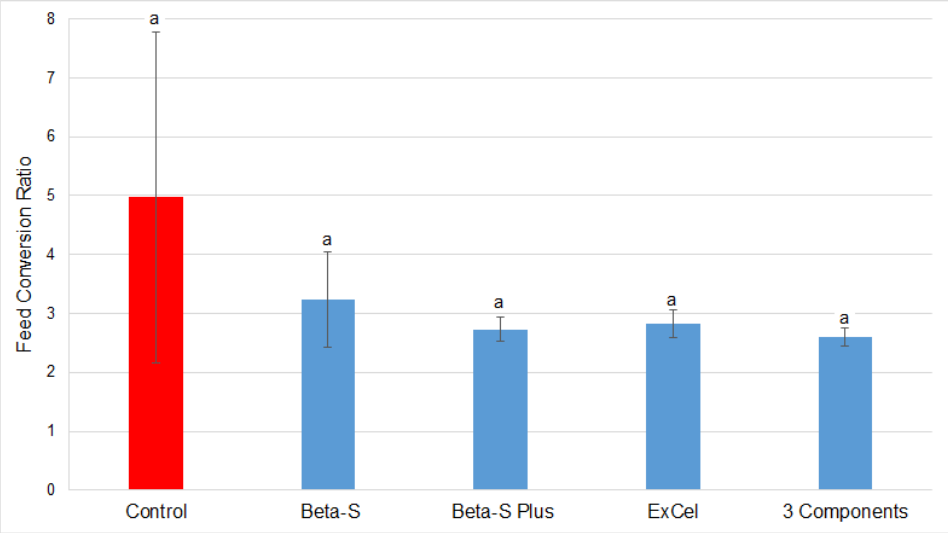


Figure 3.9: Feed Conversion Ratio (FCR) of *Litopenaeus vannamei* with four yeast derived β-glucan products and a control diet. FCR was calculated by feed intake per final net biomass, after 143 days. Data are average and similar letter means no significant differences (ANOVA + Tukey, p=0.1180).

Regarding FCR, no significant ($p>0.05$) differences were observed on the FCR among experimental groups (Fig. 3.9). The final average yield of the five experimental groups was calculated based on the final biomass minus the initial biomass (Figure 3.10), after 143 days. Treatments *B*-S Plus, ExCel, and 3 Components had a more significant yield than the control ($p<0.05$) were more advantageous than the control group. Significant differences were observed among *β* glucans treatment and control groups ($p< 0.05$)

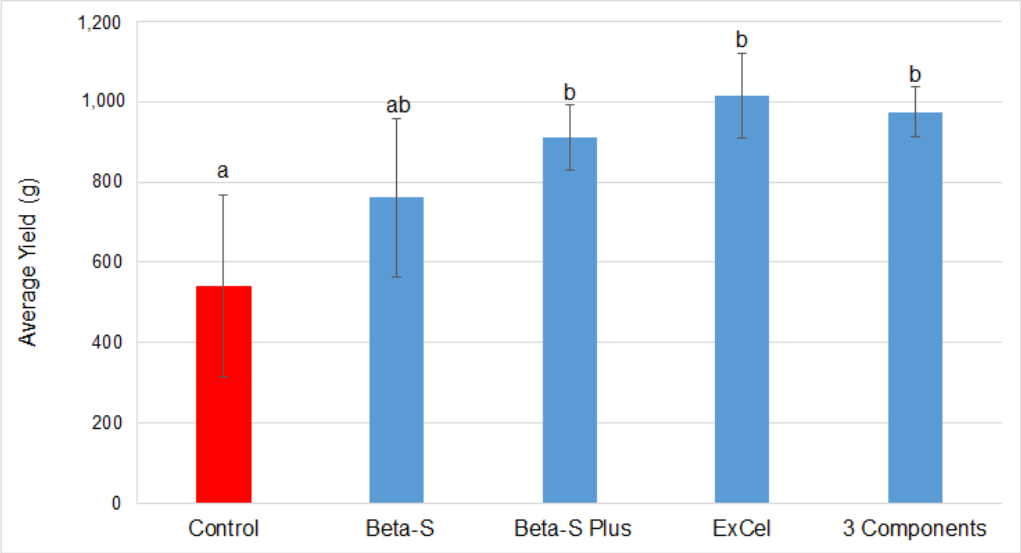


Figure 3.10: Final average yield as a *Litopenaeus vannamei* farming indicator. Shrimp received four yeast derived β-glucan diets and a control group, during 143 days. Yield per cage was computed by the subtraction of final biomass by initial biomass. Data are average and different letters mean significant differences (ANOVA + Tukey, p=0.0027).

Regarding the proximate analysis of the shrimp, tree Components resulted in a slighthy higher better protein content than control group. On the other hand, shrimp fed with Excel presented a lower level of lipids (%) compared to the control group and a lower level of energy content of all experimental groups. Table 3.3 summarizes the zootechnical parameters observed on the trial, as well as the shrimp proximate composition results. A summary of the production data and general results comparing dietary treatments and control is shown in table 3.3.

Table 3.3 Results from shrimp cultured with β-glucan and proximate composition from shrimp carcass.

Type of parameter	Control	*B*-S	*B*-S Plus	ExCel	3 Components
Total production (g.cage^{-1})	846	1062	1214	1318	1279
Final cage weight (g)	845.75±230.58^{a}	1061.50±197.39^{ab}	1213.75±81.14^{b}	1317.50±112.90^{b}	1278.50±57.35^{b}
Average shrimp weight (g)	19.50±0.87	18.85±2.18	18.33±1.15	20.23±1.60	17.91±0.87
Percentage of weight gain (%)	178±0.79	255±0.74	299± 0.17	335±0.30	320±0.22
Average shrimp weight gain (g)	16.45±0.90	15.85±2.24	15.28±1.09	17.20±1.47	14.86±0.83
Density (g/m^{3})	528.61±144.13^{a}	663.70±123.35^{ab}	758.89±50.53^{b}	823.66±70.51^{b}	799.28±35.87^{b}
SGR (on a cage basis)	0.69±0.22^{a}	0.87±0.14^{a}	0.97±0.03^{b}	1.03±0.05^{b}	1.00±0.04^{b}
FCR	4.97±2.82	3.23±0.81	2.73±0.21	2.83±0.24	2.60±0.15
Survival (%)	43.50±12.12^{a}	56.00±4.55^{ab}	66.25±2.50^{bc}	65.25±4.92^{bc}	71.50±4.36^{c}
Carcass composition from shrimp					
Protein (%)	72.53±0.68^{a}	73.90±0.89^{ab}	73.51±1.17^{ab}	73.43±0.06^{ab}	75.42±0.25^{b}
Lipid (%)	4.94±0.09^{a}	5.14±0.12^{a}	5.09±0.52^{a}	4.20±0.01^{b}	4.45±0.30^{ab}
Ash (%)	5.34 ± 0.18	5.30 ± 0.06	5.14 ± 0.19	5.99 ± 0.11	5.10 ± 0.40
Moisture (%)	98.15 ± 0.15	98.02 ± 0.15	97.93 ± 0.09	98.10 ± 0.04	98.21 ± 0.05
Energy (MJ.kg^{-1})	19.76±0.11^{a}	19.60±0.08^{b}	20.18±0.08^{b}	18.83±0.07^{c}	19.58±0.25^{b}

Different superscript letters within rows indicate significant differences ($p<0.05$) between treatments.

Overall results showed supplementation with *B*-S Plus and 3 Component ended up with better FCR compared to the control group (1). All treatments diets with β-glucans resulted with significantly higher survival rate compared to the control group and some treatments such as 3 components resulted in 71% survival (2) After 143 days rearing in open ponds resulted in satisfactory aquafeed experiment. (3) An increase in biomass was observed with in cages among all groups but significantly higher with the use of B-glucans. (4)

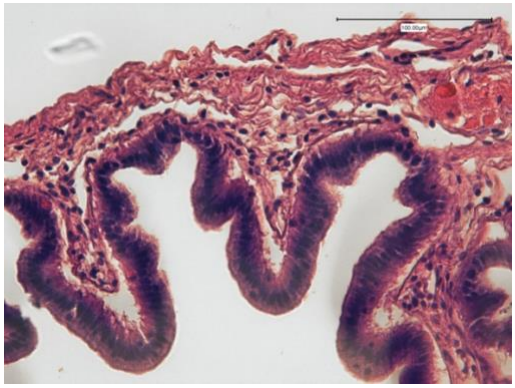
Table 3.4 Production data and general results from trials vs control.

General results from trials vs control	
Increase in production per m^3 ratio	1:1.45
Survival increase	24%
Percentage of earning per cage	35%
Increase in cost from the use of β-glucans	8 - 6%
Production data	
Production days	143
Sizes maximum and minimum (g)	18.9
Weight gain ($g.week^{-1}$)	0.94

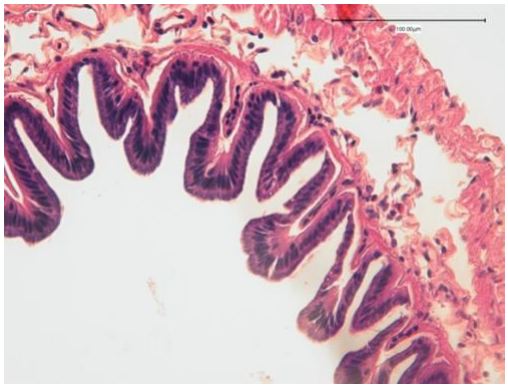
Table 3.5 Inclusion rate of different betaglucans and general characteristics from treatments

No.	Color ID	Treatment	Composition	Protein [%]	Lipids [%]	Energy [kCal/100 gr]
0	White	Blank	Control diet ABAFOR	35	8	17
1	Green	Excel	Control diet +Leiber Excel (3 kg/ton) → yeast autolysate	35	8	17
2	Blue	Beta S	Control diet + Leiber Beta S (200 gr/ton) → β-glucan	35	8	18
3	Red	Biolex	Control diet + Leiber Beta S (mix of 200 g Beta-S and 800 gr yeast cell wall product Biolex MB40, dosage 1kg/ton) → β-glucans and yeast cell wall	35	8	17
4	Yellow	3-Comp	Control diet + Leiber Beta-S Plus (1kg/ton) + Leiber Excel (3 kg/ton) → combining all 3 components (β-glucan + yeast cell wall and yeast autolysate)	35	8	18

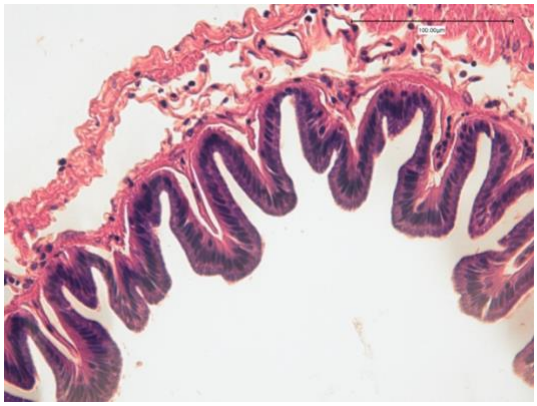
3.6 Gut morphology and shrimp histology



A) Control group - White treatment



B) Beta S Plus - Red treatment



C) Excell – green treatment

D) 3 comp – yellow treatment

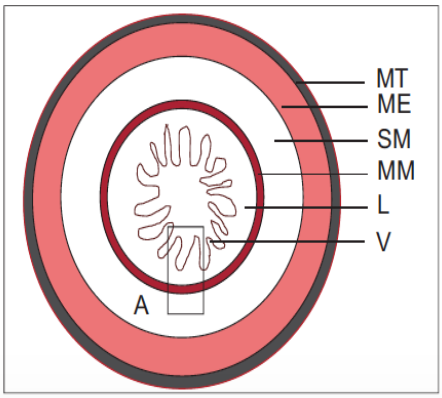


Figure 3.11. Schematic diagram of shrimp posterior gut, macrostructural gut silhouette, displaying lamina propia, mucosa and microvilli (MV) and funtional border (V) C) hepatopancreas cells from shrimp, D) representative illustration form transversal view from shrimp gut and shrimp sections.

Histology analysis was performed at University of Plymouth in where we took samples from multiple section from our experimental shrimp (anterior, mid and posterior gut as well hephatopancreas) and it was fixed and process with H&E. Unfortunately, after sampling 160 slides, no correlation was found in between, to our knowledge the samples weren't well process, it suggested to increase the analysis only in the posterior part of the shrimp for gut structure enhacement and also hepathopancreas histology when looking for inmmune hitopatholgy. Unfortunatelly no differences where fund in between treatments due to mayor concerns with taking samples, lack of experience and misinterpretation of histological samples and slides.

3.6 Economic Analysis

As mentioned before, the amount of scientific work related with β-glucans in aquaculture is quiet significant, nevertheless economic analysis and commercial scale evaluations are limited. If the intention is to promote the use of this type of products in aquafeed diets as a tool to enhance health and increase survivals, it is important to

generate well supported information adequate to the commercial circunstances, similar results are presented as part of this research work (Table 3.6).

Table 3.6. Economic analysis, cost per kilogram of shrimp in floating cages.

Feed Additive	Biomass	FCR	FAP	Total cost of feed	Larvae	Other expenses	Cost per kilogram of Shrimp	Market price per $5.5 usd	Profit
Excel	1.316	2.8	$10. 2	$2.14	$0.50	$0.50	$3.14	$7.23	$4.23
3 Comp	1.278	2.6	$21.9	$1.98	$0.50	$0.50	$2.98	$6.9	$2.98
B S	1.061	3.2	$8.22	$2.44	$0.50	$0.50	$3.44	$5.8	$2.36
B S plus	1.213	2.7	$2.05	$3.05	$0.50	$0.50	$3.05	$6.63	$3.58
Control	846.2	4.9	0	$3.73	$0.50	$0.50	$4.73	$4.61	$0.09

* FAP (feed additive price, 11-2015)
*Prices in $USD

In order to understand the economic values from the farmed shrimp with floating cages, I used farm's data from previus cycles to support performance costing under same culture conditions, market price at the time was obtain from a large retail market located in Zapopan, Jalisco, also known as Mercado del Mar. Also, in particular from leading company named Frizajal S.A de C.V (J.G. Buenrostro Inc) who operates in Zapopan, besides retain and storage services, the company operates a couple thousands hectares divided into shimp ponds near Sinaloa state, also has remarkable recognition nation wide. It's important to mention the discrepancies between farm gate prices and the Urner Barry prices. Our study is using a price list from the domestic market, as its best for this study reflecting domestic prices in particular this year after been hit by EMS dissease in 2013-2014. A standar price of $5.5 USD per kilogram of fresh shrimp at 18-gram size, head on shrimp was used for this research work (2015).

Separately, as part of the cost of production analysis, a cost unit was generated from farm general book expenses (based on the farm´s historic data, invoices) Colima's farm cost of production at the time was confirm with other farmers nearby.

The detail cost of production is normally affected by a multifactorial components and can be affected and readjusted on a day to day basics, as seen in farm's financial books, growth cruves and shrimp models.

Consulted websites were:

- http://frizajal.com.mx
- https://www.ubcomtell.com/Login/Comtell/?ReturnUrl=/CTDefault.aspx?cookieCheck=1
- http://www.fao.org/in-action/globefish/market-reports/shrimp/en/
- http://www.phillyseafood.com/shrimp-size-chart

Our results show good improvement with the positive treatments using β-glucans versus control diet and some performance parameters were improved such as survival (Table 3.6) profit was increase above 30% in most positive treatment with a significant increase in biomass, colour and size, increasing, yields, market opportunity and price simultaneously. In particular, the Beta S-Plus and Excell treatments showed an increase in profit per kilogram of shrimp at the time of harvest.

When analyzing the cost-effect of the inclusion rate, the Return of Investment (ROI) was above 10:1 which is considered for most business exceptional in selected treatments. Nevertheless, its recommended to perform a similar analysis under commercial conditions, ideal to organize a series of trials from cages, tanks to raceways and finally earthern ponds, analyzing all shrimp stages from postlarvae to harvest size, its only when we collect multiple economic data and exclude variables involved each

particular farming system when we can actually calculate a cost benefit result than can be replicated under similar conditions.

The net profit, market opportunity, cost per unit and performance gain can make a complicated interaction of varibales that can be read only when good quality data is obtain at the site, in where the cost of production is a very volatile parameter affected by numerous biological aspects (often not in the books) which are constantly evolving in positive or negative monetary values, only when the indicators are above the standards (55% survivals, FCR ~ 1.3/ 1.7, SGR > 1.2) a competitive kilogram of fresh shrimp can be produced and sold with margin.

Therefore shrimp economics must be evaluated by experts in this field, also seafood brokers and financial professionals who can forecast daily prices, demand increase and market trends, then a manager in charge will shape the farm strategy and readjust.

Separately, price forecast and market trend involves a clear understanding of the shrimp in domestic and international markets, laws, international trends, capture shrimps, illegal imports, and previously frozen inventory from years back this information is usually limited not official and blurry. I believe it can be only obtained at the processing plants, warehouses and mayor markets. Fortunatelly shrimp comes in all sizes and presentations, as seen over the years inwhere shrimp is the top rated seafood and its large consumed in the USA and many other countries like Mexico and Brazil.

(NOAA,2017, https://www.fisheries.noaa.gov/national/fisheries-united-states-2017).

Information about market price, profit and related can be found on:

- Sagarpa (*Anuario estadisticos de acuicultura y pesca*)
- *https://climapesca.org/2018/02/05/perspectiva-de-la-produccion-camaronera-de-cultivo-2018/*
- https://fishmanmkt.com/pages/market-prices
- https://www.fisheries.noaa.gov/national/fisheries-united-states-2017

3.7 Discussion

Immunostimulatory substances as dietary supplements, such as glucans. β-glucans have appeared to be convenient for use in aquaculture and have been proved to have a positive effect on the growth and survival of shrimp, and it also has been shown to

enhance the immune response and resistance to bacterial and viral infections. The modulation of the immune response, improving gut morphology, feed conversion ratio (FCR) growth is a real possibility for β-glucan in Shrimp feeds. A low level of addition of the β-glucan to shrimp feeds does not affect growth performance, in fact, it promotes higher assimilation of natural food and flow protein shrimps feed when they reach commercial size lowering FCR and increasing profits for the farmer.

The β-glucans are physiologically active compounds capable of modifying the biological response and classified as biological response modifiers (Novak; Vetvicka 2008). Thus, in order to evaluate the use of β-glucans in the shrimp farming, the present work analysed the response of the White shrimp, *L. vannamei*, to the dietary supplementation with four products or blend of β-glucan, in commercial conditions.

Based on our results, the nutritional factors of β-glucan promoted the capacity of the shrimp immune response, observed mainly in the animals that received the diets *B*-S Plus, ExCel and 3 Components, which presented greater survival and specific growth rate in relation to the control group. Immunomodulators such as β-glucan, manipulate the immune systems by stimulation of defence proteins from hemocytes to be released into shrimp circulation and then provide resistance against pathogenic microorganisms (Smith et al. 2014). In the case of the present work and based on the performance results, probably the dietary supplementation caused an immunopotentiation, i.e., positive modulation of the immune system (Novak; Vetvicka 2009). The receptors on the surface of the animal cell recognize and bind to the β-glucan, which stimulate the immune response and improve animal resistance (Meena et al., 2013). Consequently,

the growth enhancement and better survival observed can be understood as result of the improved disease resistance due to the immunostimulatory effectiveness of the β-glucan diet.

The information collected from the experiments during the 143-days trial in cages showed significant data differences between β-glucans diets and the control group. Moreover, there were three groups which were favored in relation to the percentage of survival by individuals and thus achieving a better SGR and a better profit, i.e., *B*-S Plus, ExCel, and 3 Components, additionally to a higher final biomass and better average yield, although no differences were observed on the final average weight among groups. Nevertheless, aspects as the SGR can be affected by external factors like density and biomass.

Throughput information gathered during several cycles and based on the personal expertise, we concluded that growth is directly related to the size and biomass per m^2 of shrimp, which along with the stress factors generate a negative effect on the SGR and FCR in conjunction with other parameters. Therefore, it is recommended to make a partial harvest in order to minimize the stress in the organisms caused by density or run a feeding trial for fewer days taking as parameter a density of 350 g/m^2. Despite this observation, as there were no differences in the FCR among the experimental groups, we can affirm all dietary groups presented a satisfactory nutrient utilization, with no loss in the feed conversion.

Similar results were found by Chotikachinda et al. (2008). These authors observed that *L. vannamei* fed with inactive yeast cell wall presented no significative differences in weight and growth rate, additionally to survival, but the better effect on immune parameters. Chang et al. (2000) describe that adults *Penaeus monodon* fed with β 1,3 glucan from *Shcizophyum commune* presented better survival than the control group and immunostimulatory enhancement. Further, Li et al. (2019) reported that *L. vannamei* fed with β-glucan had improved growth performance and survival even under low salinity, enhancing shrimp digestibility, antioxidant capacity, and immunity. Finally, Mohamed et al. (2017), in a study with *Macrobrachium rosenbergii* fed with commercial fed additives containing β-glucan, showed that those animals presented better shrimp performance, including survival and FCR.

Regarding the salinity, the White shrimp is a euryhaline species, tolerating an ample range of salinity, from 0.5 to 45 - 50 practical salinity unit (psu), with optimal salinity of 20 – 25 psu (New; Kutt, 2010; Roy et al. 2010). Moreover, the salinity stressful test is commonly used as a low-cost evaluation of the health status of the shrimp (Xie et al., 2018). In the present study, the low salinity system used did not affect, in any aspect, the physiology and well-being of the animals from any experimental group.

The evaluation of the nutritional properties of fish and shellfish for human consumption is of relevant interest. Shrimp provide a high quality of nutrients and are a significant source of nutritious food to humans. The nutritive values of shrimp depend upon their biochemical composition, such as protein, lipid, ash, moisture, and energy (Banu et al., 2016), all components evaluated in this study. Based on our results, shrimp fed with 3

Components diet had the most satisfactory protein content, especially in comparison to control group, while shrimp fed with ExCel diet were the lowest source of lipid and energy. Thus, shrimp fed with a diet supplemented with β-glucan have their body composition affected by the diet, presumable due to a better nutrient assimilation and accumulation in the shrimp meat. Moreover, they may present attractive nutritional properties in the proximate composition from their carcass, especially those fed with 3 Components, in terms of protein content, and ExCel, with a decrease in fat content, being a meaning source of nutritious food for the human consumers.

Mohamed et al. (2017), in a study with a commercial source of feed additives containing β-glucan, observed no differences in the proximate composition of *M. rosenbergii* carcass, although authors have observed percentual differences in protein (increase) and fat (decrease). On the contrary, Boonanuntanasarn et al. (2016) have observed that *L. vannamei* fed with β-glucan or β-glucan associated with synbiotics had higher moisture and ash content, additionally to the increase in protein content in shrimp meat.

3.7 Conclusions

1. Shrimp zootechnical performance was improved with the use of the experimental diets *B*-S Plus, 3 Components, and ExCel.

2. Nutritional content of experimental diets was enhanced with the use of the diets *B*-S Plus, 3 Components, and ExCel.

3. The experimental diets *B*-S Plus, 3 Components, and ExCel shown to be appropriate to shrimp farming, resulting in attractive animal performance.

4. The experimental diets *B*-S Plus, 3 Components, and ExCel can maximize shrimp farming in terms of economic profit.

5. β-glucan can be used as dietary supplementation for Mexican shrimp farming.

CHAPTER 4. Changes in gut microbiota with the use of Yeast & Terrestrial Herbs (YAH) as a commercial immunostimulant on the Pacific White Shrimp (*Litopenaeus vannamei)* 25/6/16-25/9/16

4.1 Introduction

4.1.1 The use of natural Immune modulators

The shrimp immune system is based on an efficient innate system, which is composed of both cellular and humoral components. In brief, the innate immune response is based on the recognition of the pathogen-associated molecular patterns (PAMPs) by the pattern recognition receptors (PRRs), which activate, through the signal transduction cascades, the humoral and cellular defences (Li; Xiang, 2013; Xu et al., 2014). In order to have an environmentally friendly and sustainable shrimp farming industry, researchers and farmers are looking for and developing new prophylactic treatments to prevent and protect animals against pathogens and also resistance to stressful situations. Thus, the contemporary approach is to find feasible substances to enhance the immune system, such as the immunostimulants, which can be administered phophylatically by injection, immersion, or orally (Smith et al., 2014). For shrimp, the presentation as a diet supplement is the most effective administration way of immunostimulants (Zhang; Mai, 2014), followed by immersion treatment, due to reduced or absence of handling stress and risk of wounding. Those treatments should be made at frequent and regular intervals in order to achieve effective protection (Azad et al., 2005), and the correct dose should be respected, due to overdosing may lead to immunosuppression (Mehana; Rahmani; Aly, 2015).

The mechanism of action of polysaccharides are related to specific binding proteins, opsonins, and other defence proteins that activate the cellular function when reacting with β-glucans or LPS (lipopolysaccharides), i.e., when the β-glucans bind to the receptors on the hemocytes, occurs the stimulation of the immune responses. The complex between β-glucans and the binding protein reacts with the hemocyte surfaces, releasing the hemocytes granules, which, in turns activates the proPO systems, the clotting cascade, and expression of genes coding for antibacterial proteins. Defence molecules are released into circulation from the hemocytes, and can active functions such as encapsulation, coagulation, melanization, phagocytosis, modulation of cytokine production and thus, counter pathogenic microorganisms in the blood or tissues (Meena et al., 2013; Karunasagar et al., 2014; Smith et al., 2014; Zhang; Mai, 2014).

4.2 Aims and objectives

The purpose of this study was primary to identify the biological potential and environmental and microbial ecological aspects relating to the composition of gut microbiota in cultured shrimps and their health status. Gut microbiota is also known as the new organ (Baquero; Nombela, 2012; McFall-Ngai et al., 2013), with an important role in digestion (physiology), nutritional metabolism and immune component (Akhter et al., 2015). The use of novel feed additives and their influence-on their gut microbiota can be a great asset to understand patterns in bacteria and environmental changes. The application of novel feed additives in aquaculture species has a long history in the field. Thus, the search to include sustainable feed ingredients

into aquafeeds, could lead to stabilization of potential beneficial bacteria in the gut and act as effective disease control agents.

4.2.1 The objectives of this chapter were therefore:

1. To estimate the influence of a Yeast and Herbs (YAH) on shrimp performance under typical cultured conditions.

2. To evaluate the modulation of bacterial communities with the inclusion of Yeast and Herbs (YAH) and some aspect of nonspecific immune response

3. To understand the role of the shrimp microbiome when fed with a novel feed additive, such as Yeast and Herbs (YAH), and comprehend the role of the most relevant bacteria phyla and genera in the shrimp gut and its correlation with nutrition and animal health.

4.3 Materials and Methods

With the aim of improving the evaluation of feed additives from yeast blends and correlated data from our first trial with β-glucans using the same methodology and new analysis technologies to correlated shrimp survivals and microecosystems, performance, nonspecific immune response and gut microbiome in commercial conditions.

4.3.1 Experimental design

In the experimental arrangement for 102 days' trial was conducted with 1,200 juvenile shrimp with average of (3.00 g ± 0.25 g size), floating cages where stoked as mentioned on previous trial and distributed randomly in a 1500 m^2 shrimp pond with HDPE liner with sand bottom. Water was pumped from an artisanal well with oceanic salinity 35 ppt with no filters and simple infrastructure use to pumped water, our experimental design included twelve cages and were stocked randomly in the pond, being four cages per treatment (Figure 4.1).

The daily assessment of water quality parameters included total salinity, ammonia, nitrite, pH, water temperature, and dissolved oxygen. Biomass analysis occurred weekly as we targeted this trial to become shorter in days and keep more data from cages and performance, twenty shrimp per cage, accounting for 40% of the total population were randomly selected with the use of nets and individually weighted at the site.

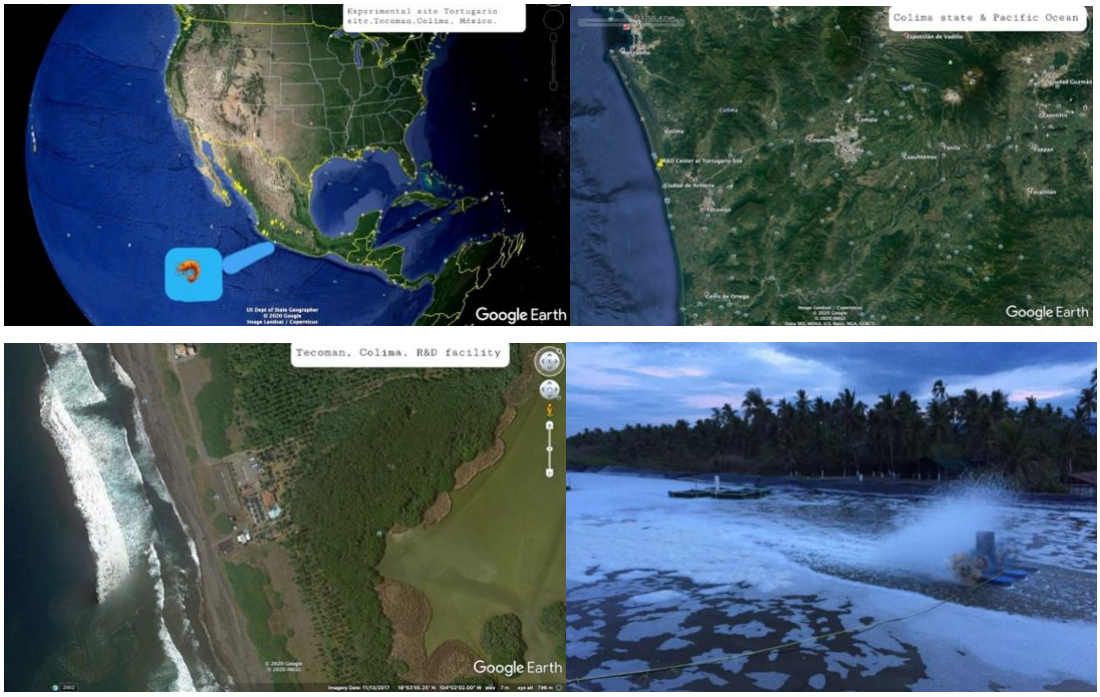


Figure 4.1: Geographic location of the "Turtle Site" experimental facility located in Cuyutlan, Colima, Mexico, by the Pacific coast (18°,53',55,25" N, 104°,02',02.20" W) Sources: https://tortugariocuyutlan.com, Google Earth 2017

4.3.2 Experimental diets and ingredients.

In this trial, two different doses of a commercial shrimp YAH (*Trichoderma longibrachiatum* powder, yeast wall extract, dry yeast from brewery, plant derivates and calcium aluminium silicate) from PAHC -USA, containing yeast cell wall and herbs, were added to the shrimp feed, and compared to a control group (without YAH supplement). A basal diet was formulated to meet the known nutrient requirements of shrimp (NRC 2011), as illustrated in Table 4.1. A thirty percent crude protein seven percent fat standard diet was formulated by Azteca Nutrition, in were four cages were fed with 5 kg of YAH per ton of feed (0.5%), four cages were fed with 10 kg of YAH per ton of feed (1.0%), while the remaining four cages were fed with commercial diet

without supplement. The YAH supplement was top coated on the pellets using soy oil (20 g / kg) as the coating agent. The control group feed was coated with soy oil devoid of the YAH supplement. Feeding rate was adjusted with the use of feeding trays and based on the farm's feeding chart recommendations, occurring twice a day. Feed quantity was adjusted according to the biomass in the cages, water temperature, and dissolved oxygen, and supported by a strong monitoring to check if feed remained on trays.

The feed for this trial was manufactured for a 30% protein, 7% fat, 2.5% fiber and 9% ash, all diets isoproteic and isoenergetic 30/7, and prepared using a 250-hp California Pellet Mill (CPM) batches of five metric tons at 2 mm size pellet. The experimental feed was prepared by mixing the feed in a mixer with the Yeast and Herbs.

Table 4.1: Feed formulation profile and ingredients source.

Ingredients ($g.kg^{-1}$)	Control	+0.5%	+1.0%
Fish meal	10	10	12
Soybean meal	22	22	22
Poultry meal	18	18	18
Wheat flour	46	46	46
Kelp hydrolysate (Binder)	0.6	0.6	0.6
Lecithin	1	1	1
Fish oil	3	3	3
Soybean oil	1	1	1
Vitamin premix b	1	1	1
Mineral premix c	1	1	1
Vitamin C	0.3	0.3	0.3
YAH inclusion	0	0.5	1
Analysis ($g.kg^{-1}$; dry weight basis)			
Moisture (%)	6	6	6
Crude protein (%)	30	30	30
Crude lipid (%)	7	7	7
Ash (%)	15	15	15

- 55/3 Soybean meal USA
- 62/6 Poultry meal, Tyson
- 7/2 Weat flour, Mx
- Kelp, Mx
- Lecitin, Mx
- Fish oil, Chile
- Soybean oils, USA
- Vitamin premix b, Vimifos
- Mineral premix c, Vimifos
- Vitamin C, Vimifos
- Antifungal, Vimifos
- Vimifos- Mexico.

4.3.3 Water quality parameters

Water quality parameters remained within the appropriate levels for shrimp farming, Throughput the trial (Table 4.2). Water temperatures were in the optimal range for shrimp culture (Figure 4.3), as well as the salinity, which was ideal for *Litopenaeus vannamei*. The oxygen levels were usually high, never lower than 4 $mg.l^{-1}$ (Figure 4.3). Values of pH were between 7.5 - 9, reaching sometimes the superior limit for culture ponds. The ammonia and the nitrite levels were low and safe. In order explain water quality we consider to take into account the minimum water exchanged involved (10% cycle) due to the small infrastructure to pump water, this condition led to an increase in organic matter within the pond, also an increase in natural productivity after week number four, secci measurements were decree by 15 –10 cm with a mixture of foam on top of the pond. Unfortunately, this condition like many others has advantages and disadvantages due to high biological demand for disolved oxygen and water parameters changing dramatically every other day. In some documents and manuals this type is system is considering a bio floc or semi biofloc.

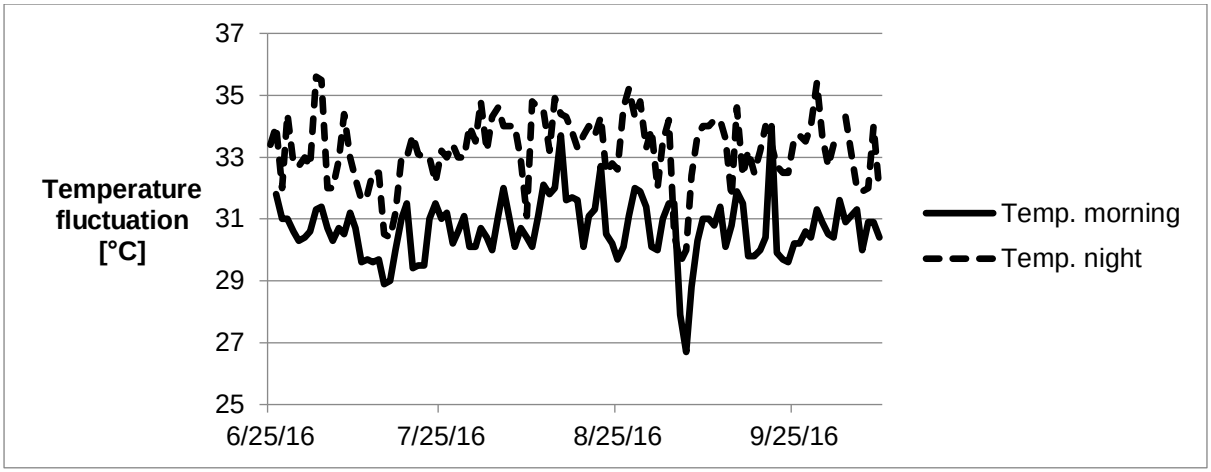


Figure 4.2 Water temperature fluctuation.

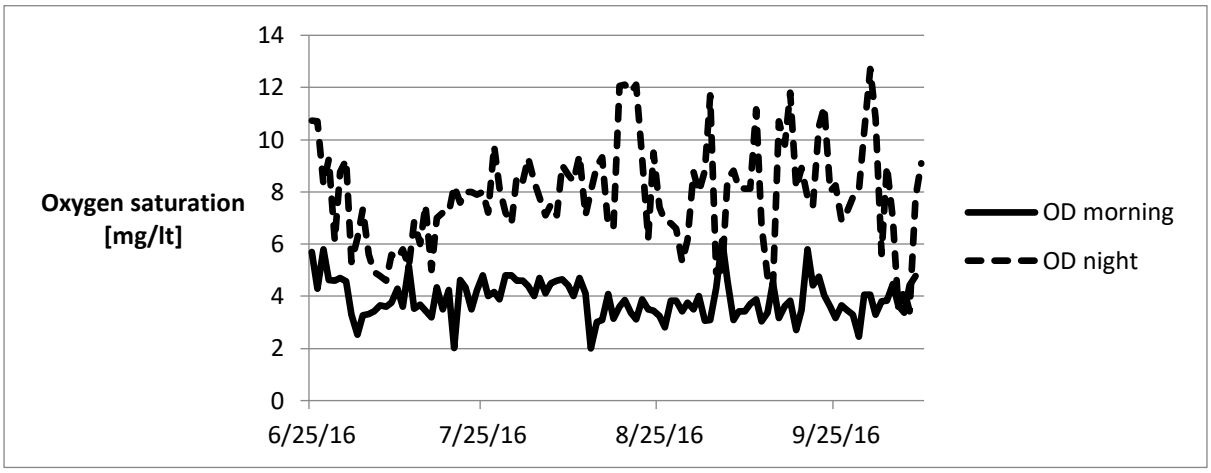


Figure 4.3 Oxygen saturation day and night measurements

Table 4.2: Water quality parameters monitored during the 102 days of experiment. Shrimp were kept in an intensive system with clear seawater and fed with two different doses of Yeast and Herbs (YAH) (0.5% and 1.0%), in addition to control group. Data are average and obtained from twice daily records.

Parameters	Results
Temperature (℃)	29 – 34
Total salinity (ppt)	33 – 35
Dissolved oxygen ($mg.lt^{-1}$)	4.0 – 10.0
pH	8.5 – 9.6
Ammonia ($mg.lt^{-1}$)	0.1 – 0.3
Nitrite ($mg.lt^{-1}$)	0.2 – 0.5

4.3.4 Sample collection

After the 102 days of trial, the cages were harvested, and shrimp individually weighted, counted, and collected for sampling (intestine). A total of 84 shrimp were randomly sampled, i.e., 28 shrimp per treatment and 7 per cage with the support of my collegue Sam Voller from Plymouth, together we set up a small site to process samples and esterized the instruments (Eppendorf tubes 25uL, 10uL, epaharine, globes, micropipette, molecular ethanol, RNA later from Sigma Aldrich), avoiding cross contamination from distinct bacterial DNA. Shrimp were euthanized by thermal shock (33 °C to 9 °C), carcass surfaces were washed with 70% (v/v) ethanol and shrimp

samples from the posterior gut with its content were collected, using sterilized tweezers and scissors. Samples were immediately fixed in 70% (v/v) molecular grade ethanol, stored in sterile 2 ml microtube, and kept under -20 ℃ for subsequent analysis. Figure 4.4 shows the map of samples and physical location of the cages. Tables 4.3 and 4.4 show the treatment, codes, labels and weights of samples during the experiment and analysis.

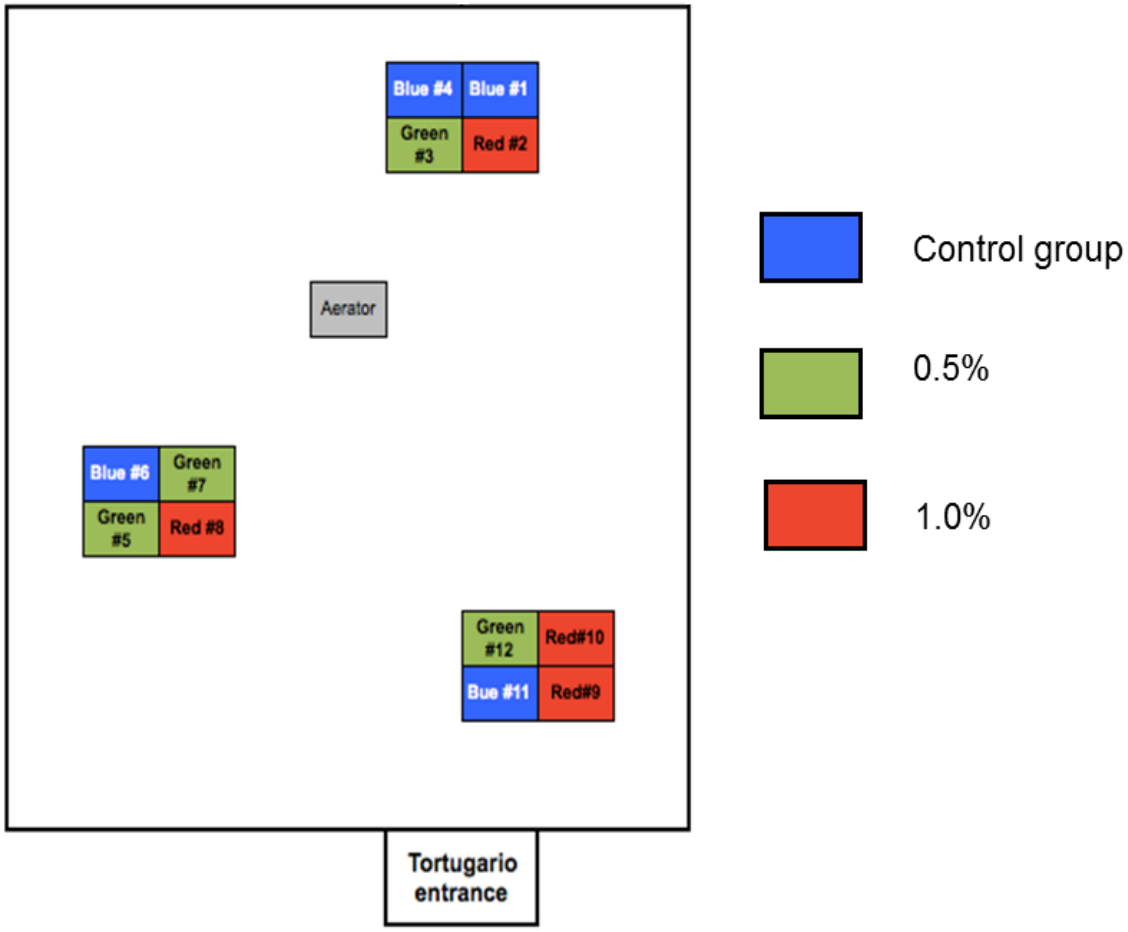


Figure 4.4: Schematic representation of samples and physical location from cages.

Table 4.3 Treatment, codes and labels.

Treatment	ID Colour	FFA inclusion [%]	Replicates
Control Treatment	Blue	0	4
Control Treatment	Green	0.5	4
Control Treatment	Red	1	4

Table 4.4: Ion Torrent runs and samples weight

SAMPLE #	CAGE CODE	Ion-Xpress Code	KS CODE	SAMPLE WEIGHT
1	C11S2	26	BLUE	30 ul
2	C11S4	27	BLUE	30 ul
3	C6S1	28	BLUE	30 ul
4	C6S5	29	BLUE	25 ul
5	C4S3	30	BLUE	25 ul
6	C4S2	31	BLUE	30 ul
7	C1S3	32	BLUE	30 ul
22	C1S2	-	BLUE	30 ul
8	C5S5	33	RED	25 ul
9	C5S2	34	RED	25 ul
10	C12S5	35	RED	30 ul
11	C12S6	36	RED	30 ul
12	C7S5	-	RED	30 ul
23	C3S4	38	RED	30 ul
30	C3S1	39	RED	30 ul
31	C7S6	46	RED	30 ul
24	C9S1	39	GREEN	25 ul
25	C10S8	40	GREEN	30 ul
26	C10S2	41	GREEN	30 ul
27	C9S4	42	GREEN	30 ul
28	C8S6	43	GREEN	30 ul
29	C8S1	44	GREEN	30 ul
32	C2S5	-	GREEN	30 ul
33	C2S6	47	GREEN	25 ul

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4.3.5 DNA extraction and 16S rRNA gene amplification

Molecular biology techniques described in this section were performed at the Microbiology laboratory at the University of Plymouth, UK. In where I performed DNA extra from 24 shrimp gut samples (n= 8 per treatment) (10 mg ± 1.5 mg in weight depeding on the sample size), using the QIAamp Stool Mini Kit (Qiagen®), and following manufacturer's instructions. In order to enhance the lysis of Gram-positive bacteria, an initial incubation with 50 mg/ml^{-1} of lysozyme for 30min at 37 ^{o}C was added to the protocol. Extracted DNA purity and quantity were measured using a UV spectrophotometer (NanoDrop™ 2000 Spectrophotometer, ThermoFischer Scientific®), based on the ratio of absorbance at 260/280 nm and 260/230 nm.

A fragment of 350 bp from the hypervariable V1-V2 regions, from bacterial 16S ribosomal RNA (16S rRNA), was amplified through a *touchdown*-polymerase chain reaction (PCR) assay, using the primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and a pool of primers 338R-I (5'GCW GCC TCC CGT AGG AGT 3') and 338R-II (5' GCW GCC ACC CGT AGG TCT 3') (Gajardo et al., 2016). For the *touchdown*-PCR, 1 µl of the DNA template (1 ng/µl) was added to the PCR mix solution containing 25 µl of MyTaq™ Red Mix (Bioline®), 1 µl of each primer (25 pM), and ultrapure DNase free water for a final volume of 50 µl. The amplification cycling profile is presented in Table 4.5. To demonstrate an accurate PCR performance, positive (*Escherichia coli* DNA) and negative (ultrapure water) controls were used in each amplification reaction. Subsequently, the amplified products were analysed by 1.5% agarose gel

electrophoresis, with SYBR Safe DNA gel stain (ThermoFischer Scientific®), in TAE buffer at constant voltage (80 V) for ~ 40min and visualized under UV light.

Pooled PCR products were purified using AMPure XP (Beckman Coulter®), based on the magnetic bead's technology. Finally, purified PCR products were sent to Systems Biology Centre of University of Plymouth UK, Genomics Facilities, for the High Throughput Sequencing (HTS), utilizing Life Technologies Ion Torrent™ Personal Genome Machine™ System (ThermoScientific®).

4.3.6 High Throughput Sequencing Results

Raw sequence data were trimmed using FASTX-Toolkit (Hannon Lab), and sequences with low-quality scores (Q < 20) were filtered out. Data were then assessed using Quantitative Insights into Microbial Ecology (Qiime 1.8.0) (Gajardo et al., 2016). Sequences were analysed using QIIME 1.8.0, and Operational Taxonomic Units (OTUs) were sorted and filtered with 97% of sequence identity. Ribosomal Database Project (RDP) tool was used to assign taxonomic affiliation, with 0.8 of confidence. Alpha and *β* diversity were calculated with ape, vegan, and R. Bacterial richness and diversity were determined with indexes such as Chao1, Observed Species and Phylogenetic diversity. Good´s coverage was also identified. Additionally, Weighted and Unweighted UniFrac distances were used to estimate similarity and dissimilarity and confirmed with Principal Component Analysis (PCoA). The taxonomic analysis was estimated with relative abundance graphs at phylum and genus level. LEfSe (Linear discriminant analysis effect size) tool was used to determine differentially abundant taxa between

treatments, and significantly different taxa were used to calculate linear discriminantl analisys LDA effect size (Segata et al., 2012), with a significant P value < 0.05 and effect size threshold of 2. Finally, the Venn diagram was built to identify the core microbiota, as well as unique and shared OTUs between treatment, using Venny 2.1 software (http://bioinfogp.cnb.csic.es/tools/venny/, Oliveros 2007-2015). Data are presented as mean ± SD. The p-value < 0.05 was considered statistically significant.

4.4 Results

4.4.1 Zootechnical performance and feed efficiency

Final shrimp survival, calculated at the end of the trial after 102 days, was statistically different between experimental groups (Fig. 4.5). Both treatments groups feed diets containing YAH displayed significantly better survival (p=0.0043) compared to the control diet. Mean shrimp survival levels were 46%, 65%, and 69%, for control, 0.5%, and 1.0% treatments, respectively.

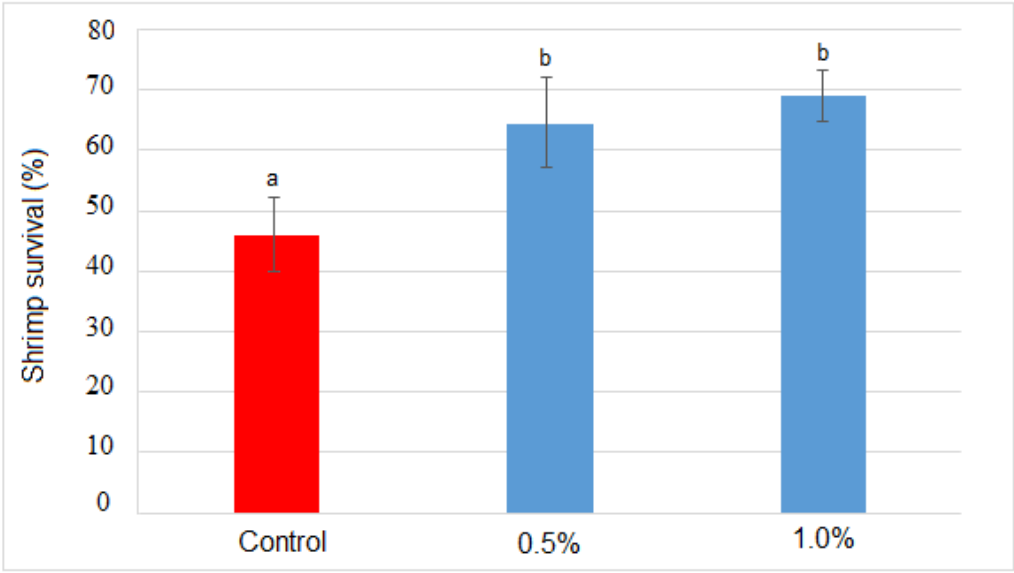


Figure 4.5: *Litopenaeus vannamei* survival with two feed doses of Yeast and Herbs (YAH) and control group. Data are average and were calculated at the end of the trial after 102 days. Different letters mean significant differences (ANOVA + Tukey, p=0.0043).

To measure shrimp final average body weight, animals were weighed individually at the end of the trial, after 102 days (Fig. 4.6). In addition, specific growth rate (SGR) was calculated based on the initial and final weight of shrimp (Fig. 4.7). Animals that received 1% of YAH showed the highest final average weight, as well as the best SGR, being both parameters statistically different from the control group. Regarding the FCR, animals from both experimental groups, i.e., 0.5% and 1.0% of YAH showed better result (Fig. 4.8).

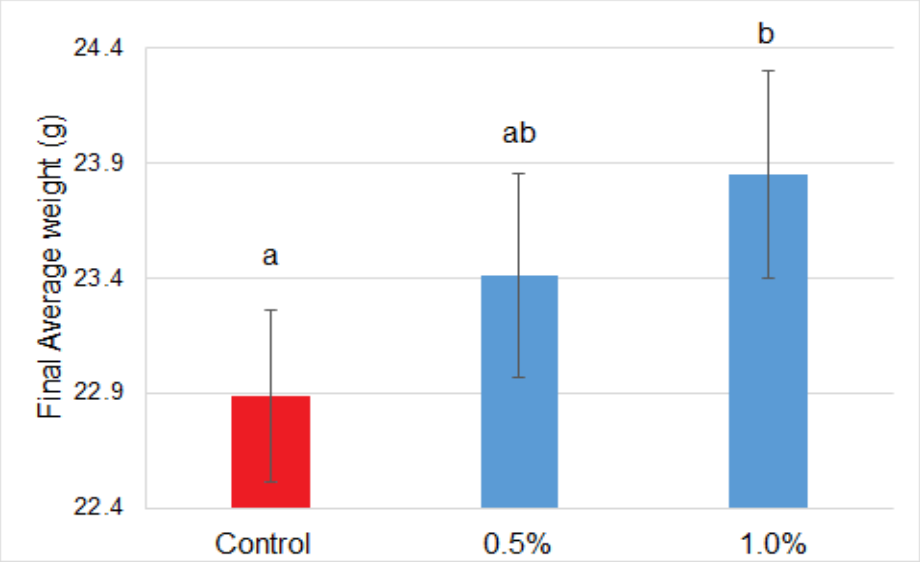


Figure 4.6: *Litopenaeus vannamei* final body weight in reference to two feed doses of Yeast and Herbs (YAH) and control group. Data are average and shrimp were weighed individually at the end of the trial, after 102 days. Different letters mean significant differences (ANOVA + Tukey, p=0.0318).

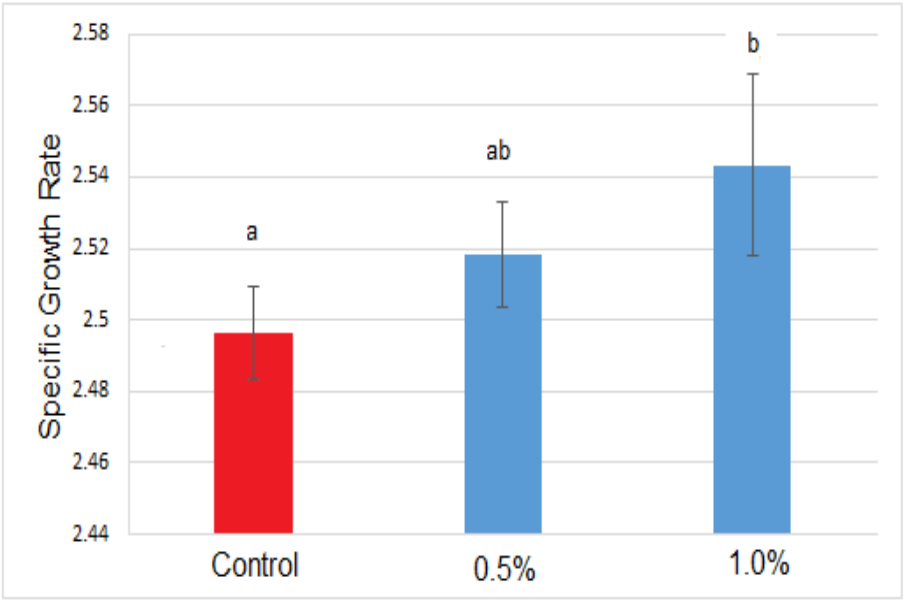


Figure 4.7: Specific Growth Rate (SGR) of *Litopenaeus vannamei* related to two feed doses of Yeast and Herbs (YAH) and control group. SGR was estimated based on initial and final shrimp weight, after 102 days. Data are average. Different letters mean significant differences (ANOVA + Tukey, p=0.0180).

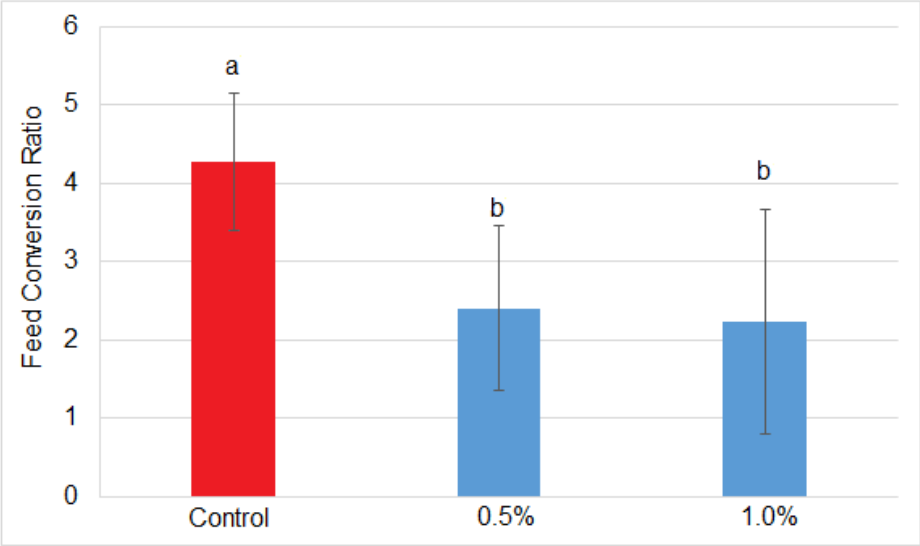


Figure 4.8: Feed Conversion Ratio (FCR) of *Litopenaeus vannamei* with two feed doses of Yeast and Herbs (YAH) and control group. FCR was calculated by feed intake per final net biomass, after 102 days. Data are average and different letters mean significant differences (ANOVA + Tukey, p=0.0006).

In order to estimate the final yield of the three experimental treatments (Figure 4.9), the yield per cage was calculated by ascertaining the net biomass at the end of the trial and the final biomass minus the initial biomass, after 102 days. In both treatments with FFA, the final yield was significantly most advantageous in comparison to the control group.

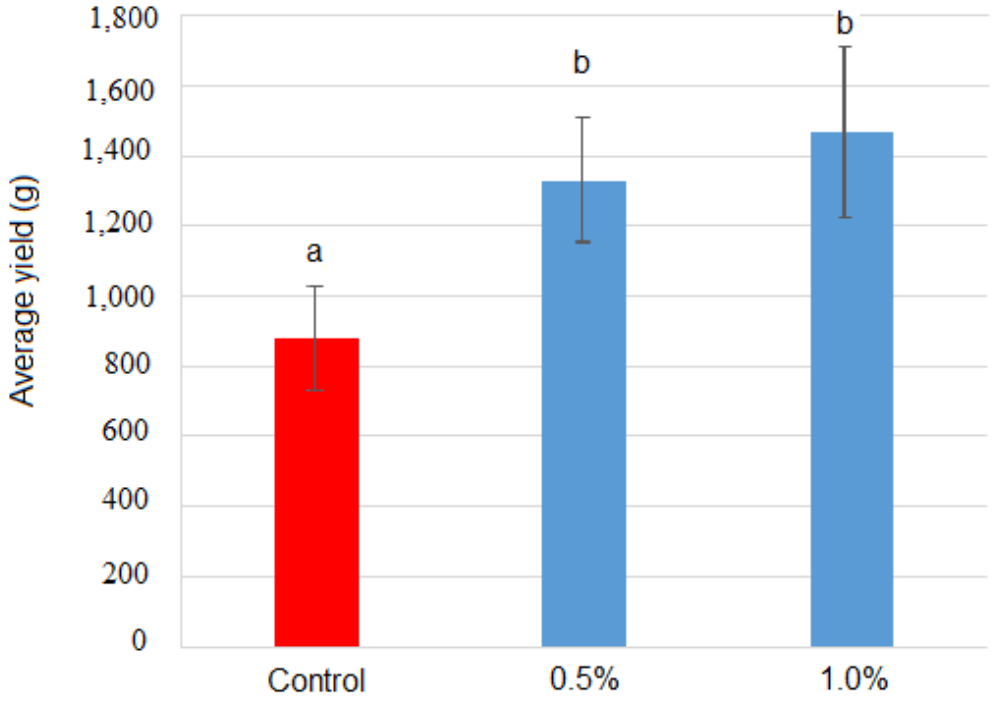


Figure 4.9: Final yield as a *Litopenaeus vannamei* farming indicator. Shrimp received two feed doses of Yeast and Herbs (YAH) and control group, during 103 days. Yield per cage was computed by the subtraction of final biomass by initial biomass, after 103 days. Data are average and different letters mean significant differences (ANOVA + Tukey, p=0.0008).

4.4.2 High Throughput Sequencing Results

The intestinal microbiota profile of *Litopenaues vannamei* was analyzed based on the sequencing of the 16S rRNA target gene, using Ion Torrent™ technology, in two different feed doses of YAH, that is, 0.5% and 1.0%, plus control group. Sequencing resulted in a total of 5,161,933 raw sequences which, after sorting and filtering, 1,983,537 were qualified as high quality. Good´s estimator of coverage presented values above 0.992, demonstrating that almost the entire bacterial diversity was identified. The rarefaction curves revealed that a satisfactory sequencing coverage was achieved, with signs of saturation for all experimental groups (Fig. 4.10). Table 4.6 summarizes the High Throughput Sequencing results.

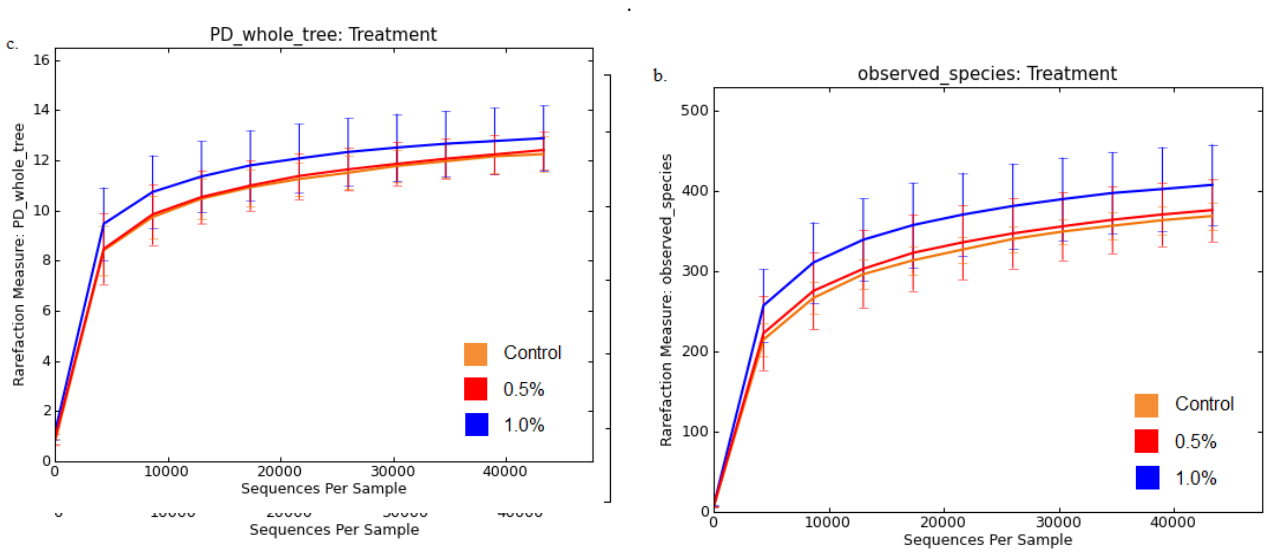


Figure 4.10: Refraction curves for (a.) Chao1; (b.) Observed species: and (c.) Phylogenetic Diversity Indexes.

Table 4.5 Summary of High Throughput Sequencing result, showing the alpha diversity indexes of *Litopenaeus vannamei* intestinal microbiota.

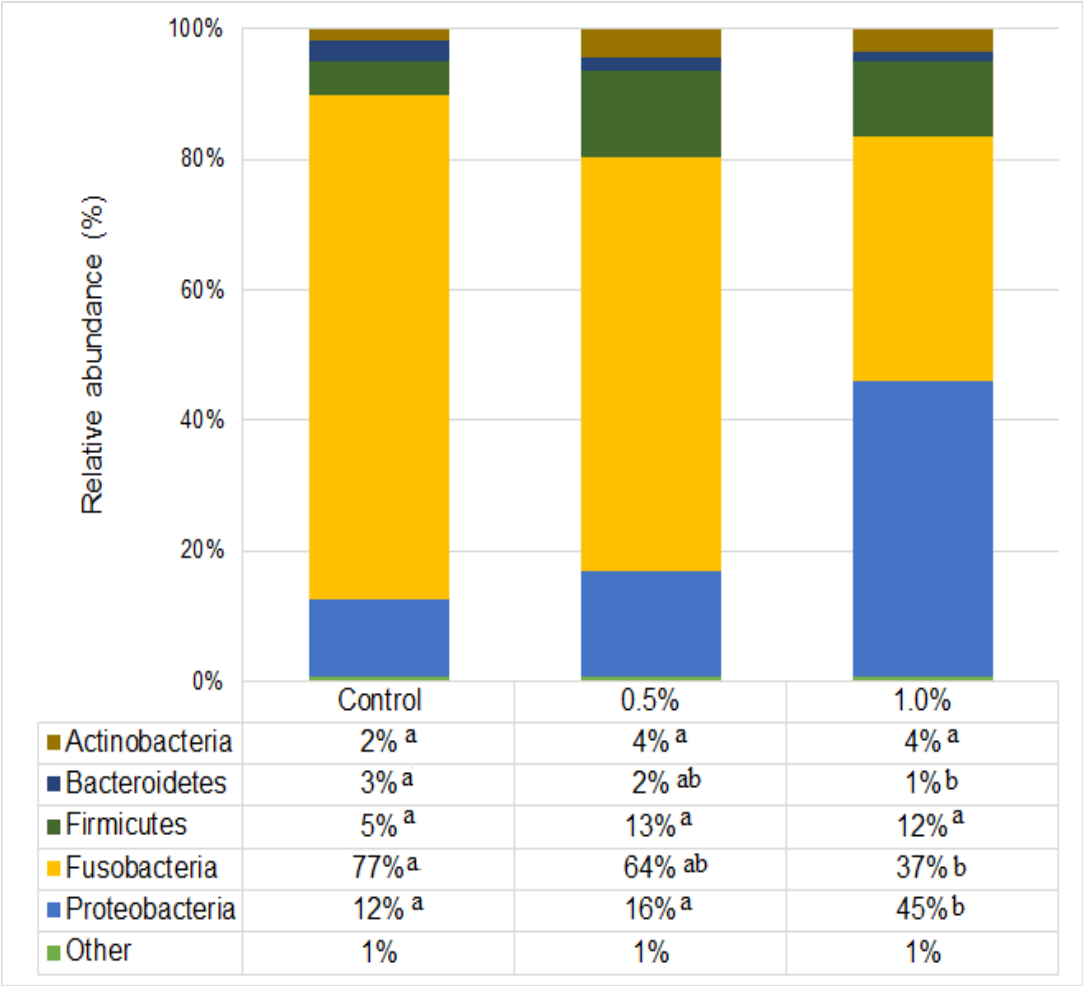
	Control	0.5%	1.0%
Reads after trimming	94,038 ± 9829	85,784 ± 5125	91,199 ± 5636
OTUs – Phylum level	15	15	16
OTUs – Genus level	194	202	206
Indexes			
Chao 1	419.53 ± 20.09	426.36 ± 26.89	452.84 ± 44.29
Observed Species	368.67 ± 17.00	375.98 ± 39.04	407.45 ± 50.45
Phylogenetic diversity	12.24 ± 0.70	12.40 ± 0.75	12.87 ± 1.30

*Indices in samples at a dissimilarity level of 3%.

4.4.3 Relative abundance phylum level

Among the five most abundant phyla in the taxonomic analysis (Fig. 4.11), the phyla Fusobacteria and Proteobacteria were the most prominent. Fusobacteria phylum was the most abundant in the gut of shrimp that received 0.5% of YAH and in those from the control group, being control group statically higher than 1.0% treatment ($p=0.0029$). The intestinal microbiota of animals that received 1.0% of YAH presented a high relative abundance of phyla Proteobacteria and Fusobacteria, being Proteobacteria relative abundance being significantly higher than 0.5% and control groups ($p=0.0073$).

These two bacterial phyla comprised had relative abundances greater than 80% in all the three analysed groups, i.e., 80%, 82%, and 89% in 0.5% and 1% treatment, and control group, respectively. Finally, the relative abundance of Bacteroidetes was statistically lower in 1% treatment than in control group ($p=0.001$).

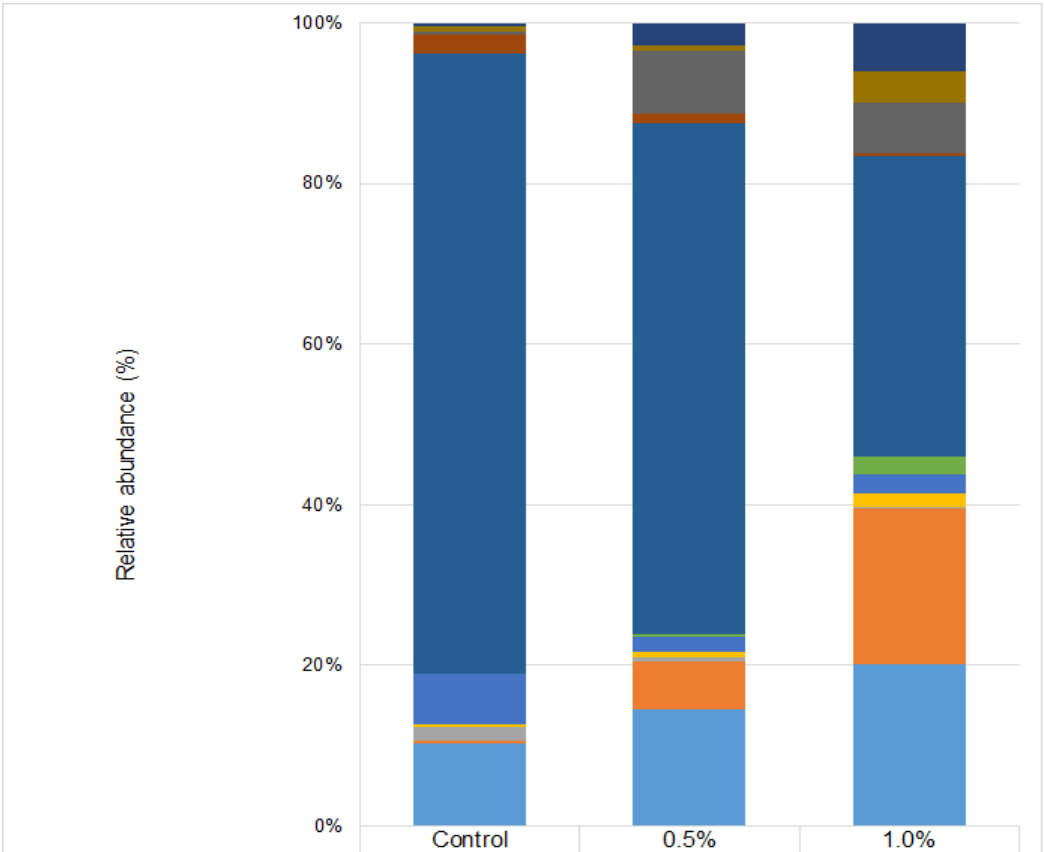


	Control	0.5%	1.0%
Actinobacteria	2% ^{a}	4% ^{a}	4% ^{a}
Bacteroidetes	3% ^{a}	2% ^{ab}	1% ^{b}
Firmicutes	5% ^{a}	13% ^{a}	12% ^{a}
Fusobacteria	77% ^{a}	64% ^{ab}	37% ^{b}
Proteobacteria	12% ^{a}	16% ^{a}	45% ^{b}
Other	1%	1%	1%

Figure 4.11: Relative abundance of gut microbiota composition of *Litopenaeus vannamei* receiving two different doses of YAH and control group, describing the distribution (%) of bacteria, at the phylum level. ANOVA + Tukey test ($p<0.05$).

Similarly, among the ten most abundant genera, the three genera *Cetobacterium*, *Sphingobium*, and *Bacillus* were distinctive (Fig. 4.12) and, summed, represent 77%, 63%, and 78% of the total relative abundance at genus level on 0.5%, 1%, and control groups, respectively. Moreover, statistical differences were more constantly observed in the 1% treatment. *Cetobacterium* was the most abundant genus of all and in all the treatments, and the 1% treatment showed a statistically lower relative abundance of this genus in comparison to the two other treatments ($p=0.0029$). Then, *Sphingobium*

was the second most abundant genus in the gut microbiota of animals from 1% treatment, which presented a higher relative abundance in comparison to the control group. *Sphingobium* was significantly higher in both treatments with FFA, in comparison to the control group (p=0.0058). Lastly, *Bacillus* was significantly higher in both treatments with YAH, in comparison to the control group (p=0.0253).



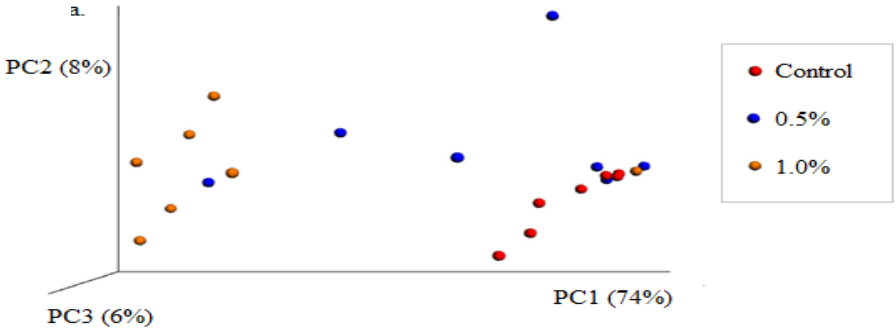
	Control	0.5%	1.0%
Acinetobacter	0.38% ^{a}	2.79% ^{ab}	6.06% ^{b}
Aeromonadaceae (family)	0.64% ^{a}	0.75% ^{a}	3.86% ^{a}
Bacillus	0.43% ^{b}	7.74% ^{a}	6.37% ^{a}
Bacteroidales (family)	2.30% ^{c}	1.25% ^{a}	0.23% ^{b}
Cetobacterium	77.31% ^{a}	63.62% ^{ab}	37.46% ^{b}
Devosia	0.01% ^{a}	0.32% ^{ab}	2.24% ^{b}
Enterobacteriaceae (family)	6.27% ^{a}	1.87% ^{a}	2.28% ^{a}
Pseudomonas	0.24% ^{a}	0.60% ^{a}	1.77% ^{b}
Shewanella	1.78% ^{a}	0.60% ^{ab}	0.26% ^{b}
Sphingobium	0.37% ^{a}	5.89% ^{ab}	19.38% ^{b}
Other	10.26%	14.55%	20.09%

Figure 4.12: Relative abundance of gut microbiota composition of *Litopenaeus vannamei* receiving two different doses of Yeast and Herbs (YAH) and control group, describing the distribution (%) of bacteria, at the genus level. ANOVA + Tukey test ($p<0.05$).

4.4.4 Similarities and dissimilarities of bacterial population

Concerning the similarities and dissimilarities of the bacterial population, the PCoA revealed a spatial separation between the categories, principally between control and 1.0% treatment. Weighted UniFrac distance (Fig. 4.13a) showed that 1.0% treatment samples clustered all together, with one exception, in the opposite direction of all control samples, while 0.5% treatment samples were dispersed. Unweighted UniFrac distance (Fig. 4.13b) displayed similar results, with spatial differentiation between control and 1.0% treatment, and with five of seven 0.5% samples clustering close to control.

a. Weighted



b. Unweighted

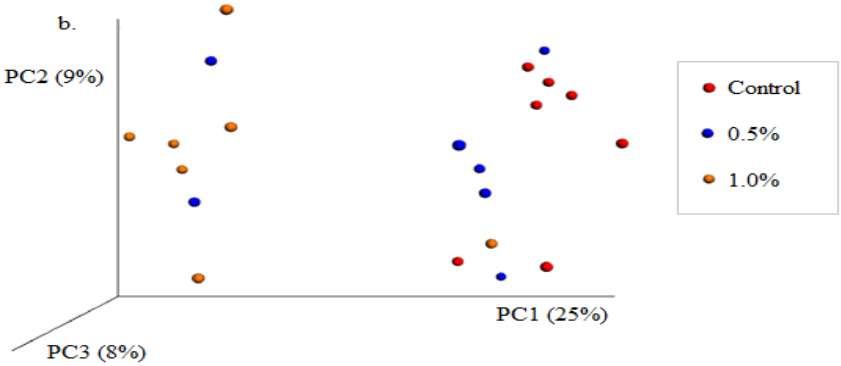
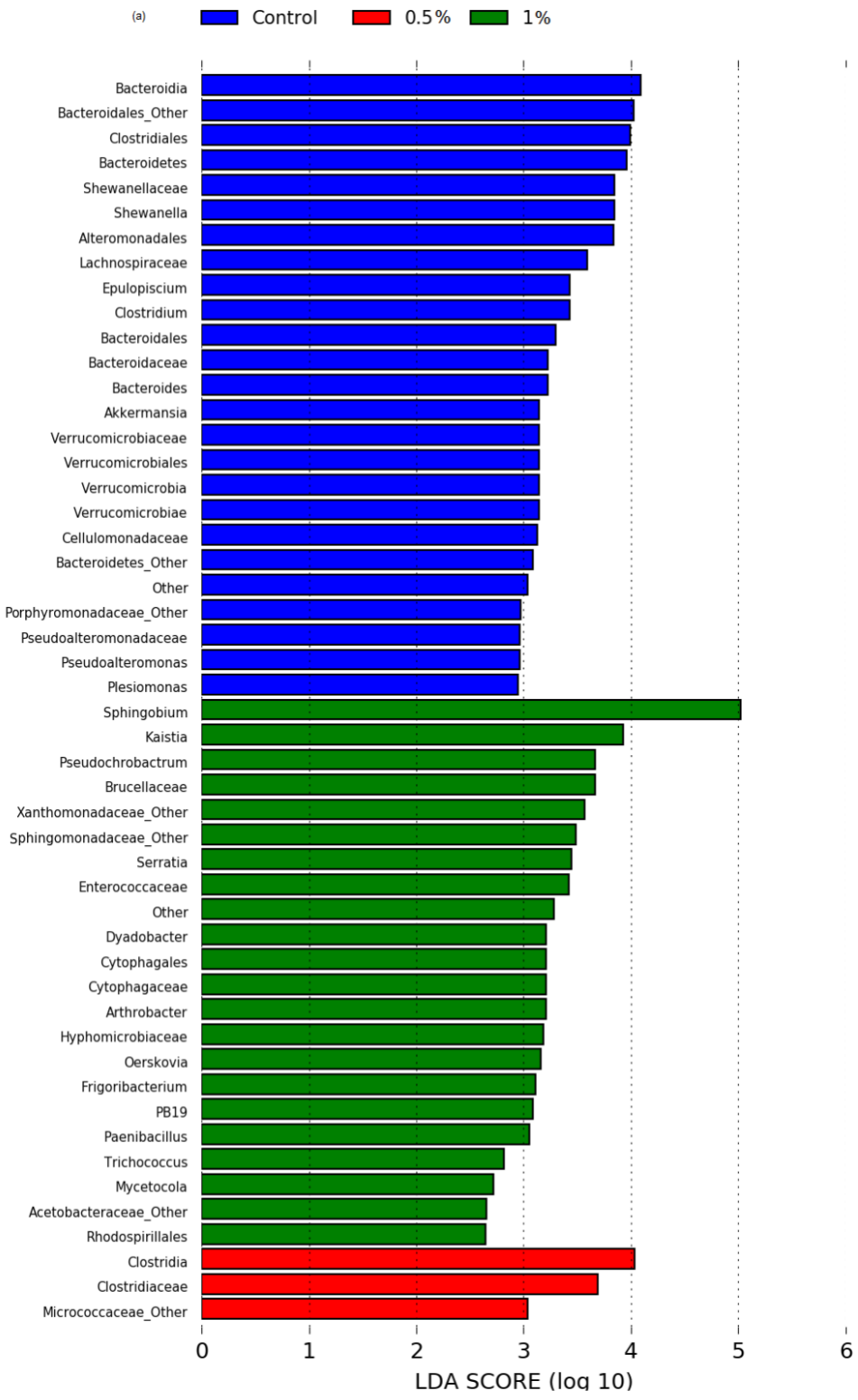


Figure 4.13: Similarities and dissimilarities of gut microbiota composition of *Litopenaeus vannamei* receiving two different doses of Yeast and Herbs (YAH) and control group, based on (a.) Weighted (percent variation explained 88%) and (b.) Unweighted (percent variation expWlained 42%). UniFrac distances.

4.4.5 LDA score and LEfSe

The Linear discriminant analysis effect size (LEfSe) is a method to discover biomarkers, identifying possible taxa that have statistical significance and biological relevance in a given population, characterizing these and showing the most likely bacteria group that can explain the differences between treatments. Regarding possible distinct taxa with statistical significance and biological relevance, the linear discriminant analysis effect size (LEfSe) identified 25 distinct taxa in control group, three on 0.5% treatment, and 22 on 1.0% treatment that could explain differences among treatments (Fig. 4.14).

Similarly, the logarithmic LDA score measures the number of differences in the relative abundance between taxa, i.e., the effect size of each feature, sorting the differences between classes of the analysed data (Segata et al., 2011) with positive scores ranging between 2.0 and 5.0. The gut microbiota of 1.0% YAH treatment showed the highest LDA score (5.0) with *Sphingobium* genus, also notable on the relative abundance at the genus level. Further, this treatment showed the smallest LDA score, though always above 2.0. Control group displayed the greatest quantity of distinct taxa, 25 in total, all above or close to 3.0 LDA score, highlighting *Shewanella* and *Clostridium* genera. Finally, 0.5% YAH treatment revealed only three distinct taxa, with LDA score between 3.0 and 4.0.


(a)
Control
0.5%
1%
Bacteroidia
Bacteroidales_Other
Clostridiales
Bacteroidetes
Shewanellaceae
Shewanella
Alteromonadales
Lachnospiraceae
Epulopiscium
Clostridium
Bacteroidales
Bacteroidaceae
Bacteroides
Akkermansia
Verrucomicrobiaceae
Verrucomicrobiales
Verrucomicrobia
Verrucomicrobiae
Cellulomonadaceae
Bacteroidetes_Other
Other
Porphyromonadaceae_Other
Pseudoalteromonadaceae
Pseudoalteromonas
Plesiomonas
Sphingobium
Kaistia
Pseudochrobactrum
Brucellaceae
Xanthomonadaceae_Other
Sphingomonadaceae_Other
Serratia
Enterococcaceae
Other
Dyadobacter
Cytophagales
Cytophagaceae
Arthrobacter
Hyphomicrobiaceae
Oerskovia
Frigoribacterium
PB19
Paenibacillus
Trichococcus
Mycetocola
Acetobacteraceae_Other
Rhodospirillales
Clostridia
Clostridiaceae
Micrococcaceae_Other
0
1
2
3
4
5
6
LDA SCORE (log 10)

b.

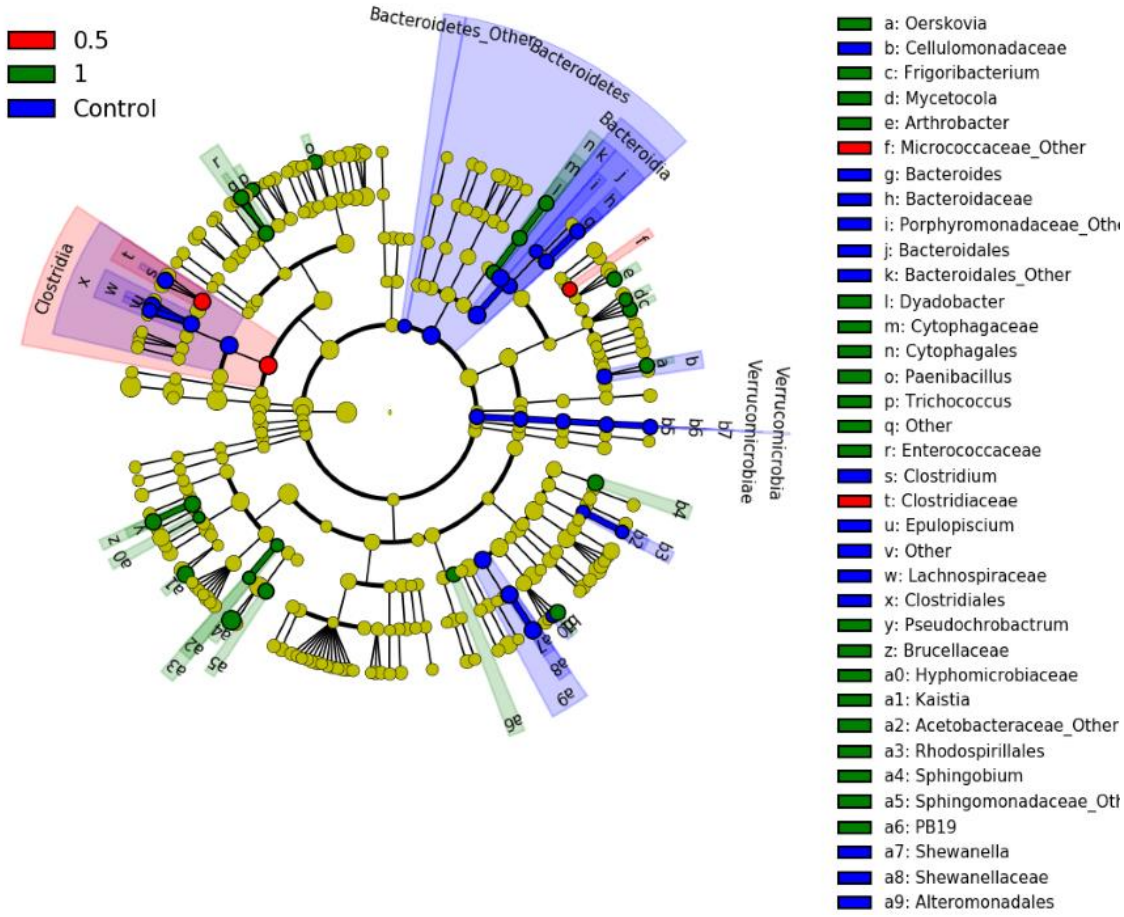


Figure 4.14 Distinct enriched taxa in the gut microbiota of *Litopenaeus vannamei*, with two different doses of Yeast and Herbs (YAH), i.e., 0.5% and 1.0%, and control group, at genus level. (a) LDA score indicating the scale of difference among taxa (b) Relative abundance of the five most abundant bacteria, at genus level, in order to support LefSe results. P<0.05; LDA threshold of 2.

4.4.6 Venn diagram and shared OTUs

The Venn diagram is a valuable analytical tool frequently used in biological sciences. The diagram permits quantitative and discriminatory analysis of differences and similarities among different groups, revealing, for instance, the core microbiota, shared and unique OTUs.

In order to define the intersection list of operational taxonomic units OTUs between treatments, such as the core gut microbiota of *L. vannamei*, as well as to determine the unique OTUs of each group, a Venn diagram was constructed, at the genus level (Fig. 4.15). The core gut microbiota comprised 57.5% of all identified OTUs, including lactic acid bacteria (LAB), such as *Lactobacillus* and *Lactococcus*, and well-established potential probiotics, like *Bacillus, Shewanella, Pseudomonas,* and *Halomonas. Cetobacterium* was also observed in all treatments. Notably, the core microbiota can be understood as the persistent microorganisms of the intestinal microbial community, i.e., the genera cited above were permanent and stable, regardless of the inclusion or not of YAH in shrimp diet.

Conversely, a number of unique OTUs were observed in each treatment. In future repetitions, the number of unique OTUs are not supposed to be the same. However, in comparable studies, abundance patterns are expected to be similar, as well as the possible biomarkers for the beneficial dietary of immunostimulants. The 1% YAH treatment had the highest quantity, accounting for 9.2% of the total OTU's within the treatment. The control group presented 1.9% unique OTUs and the 0.5% presented 3.4% unique OTU's. Additionally, when observing the intersection between the 0.5%

and 1.0% YAH treatments (0.5% ∩ 1.0%), two genera were distinct due to their potential and well-known probiotic effects, respectively *Exiguobacterium* and *Vibrio.* Therefore, shrimp aquafeed with YAH not only preserved relevant bacteria genera in the gut microbiota but also selected for two other beneficial ones. Lastly, 31.9% of the OTUs were found exclusively on the gut microbiota of shrimp feed with YAH, i.e., 66 OTUs were influenced and selected by the inclusion of YAH in shrimp feed.

Figure 4.15: Venn diagram showing unique and shared OTUs (Operational Taxonomic Units) in the gut microbiome of *Litopenaeus vannamei*, with two different doses of Yeast and Herbs (YAH)), i.e., 0.5% and 1.0%, and control group, at genus level.

4.5 Discussion

A high priority for contemporary aquaculture is to find efficient and safe technologies for prophylaxis and stimulation of animal immune system, capable of not only improving the immune system against pathogen but also to promote animal health and, consequently, better aquaculture production would be archieved. The immunonutrition applied to aquaculture, including shrimp farming, increases the immune resistance of animals and improves shrimp performance by selecting functional nutrients or functional feed additives and adding them into the aquafeed.

Furthermore, one of the critical points for shrimp farming is the image of its product and the consumer perception. The current consumer of seafood request products that are free of chemical or pharmacological residues, as well as products that originate from an eco-friendly production system. Moreover, this consumer may even pay more for a product that they judge to be superior in quality. On the other hand, they may stop consuming it due to a perception of 'antibiotic contamination' or 'environmental poisoning' (Smith, 2014). Therefore, the use of new prophylactic alternatives such as the natural compounds found, for example, in yeasts and herbs, is promising not only for the animal farming itself but also favourable to the final product image, to both local consumer and international market.

The purpose of the present study, therefore, was to evaluate the influence of a commercial immunostimulant composed of yeast cells and terrestrial herbs on the growth and health parameters, as well as on the gut microbiota composition of

Litopenaeus vannamei raised on an intensive system, simulating a commercial shrimp farming.

Primarly, the main aim of shrimp feeding trial is to achieve an optimal animal performance through evaluationingredients and products, obviously feed management greatly influences shrimp survivability. Hence, the introduction of any new ingredient in aquafeed should be critically evaluated under critical conditions and representing practical scenarios. Regarding the zootechcnical parameters, the superior performance observed for shrimps that received the YAH may have resulted from the direct induction of the immune system by the polysaccharide-rich feed, i.e., the β-glucan from the yeast cell wall, in association with the distinct compounds from the terrestrial herbs. Particularly, in our study, the synergistic blend of the natural compounds found on yeast and on terrestrial herbs maximized the shrimp production.

It is also worthy to address that, from the 16th day of trial, a heavy rain episode occurred for eight days (see figure 3.5) and, consequently, some pond parameters as temperature, BOD (Biological Oxygen Demand) were affected. This incident could have affected the shrimp survival, physiological response, nevertheless after the rain the crustaceans shown to be healthy (without any visible clinical signs or behaviour modification), good appetite and good color, with no mortalities, suggesting a good shrimp fitness due to healthy nutrition and conditions.

All the zootechnical parameters analysed were optimal for shrimp farming standards. Moreover, animals that received YAH supplementation, especially those on the 1%

YAH, presented better performance than the control group. Both treatments groups fed diets containing YAH displayed significantly better survival ($P<0.05$) compared to the control diet. Mean shrimp survival levels were 46%, 65% and 69% for control, 0.5% and 1.0% treatments, respectively.

Additionally, when analyzing the growth parameters (i.e., the growth rate and the final weight), animals that received 1% t feed additive showed better performance than those that received 0.5% or from the control group. It is recognized that the dose and the frequency that the immunostimulants are administrated can notably influence animal response (Zhang & Mai, 2014).

It should be mentioned that Sajeevan et al. (2009) studied various doses and different feeding intervals of an insoluble glucan extracted from a marine yeast in the diet of *Penaeus indicus* infected with white spot syndrome virus (WSSV). These Authors concluded that a specific combination of dose and frequency (4 to 8 servings per day) were decisive for a better shrimp survival similarity. Bai et al. (2014) reported that *L. vannamei* fed with 0.1% of carboxymethylglucan in the diet showed the best immunity and survival in experimental infection of WSSV; authors tested 18 different diets, including different β-glucan derivatives and degrees of substitution. Moreover, shrimp immunity was kept at high levels for up one month when carboxymethylglucan was added in animal feed. Particularly, the effect of the immunostimulants in shrimp tends to be transitory, thus special attention should be given to a regular frequency and the best dose (Smith et al., 2014).

The capacity of the phytobiotics to stimulate the secretion of some digestive enzymes, acting as a growth promoter (Jana et al., 2018), may also have contributed to the superior zootechnical parameters observed on animals that received YAH. Herb extracts may also increase the bioavailability of nutrients, leading to a enhance growth rate and FCR observed in our research. Several herbal products, such as *Hygrophila spinosa, Withania somnifera, Zingiber officinalis, Solanum trilobatum*, among others, have growth promotion, anti-stress, immunomodulation, and antimicrobial activities (Citarasu, 2009). In post-larvae of *P. monodon*, the papain enzyme present inon papaya leaf meal could increase protein digestion, FCR, SGR, and weight gain of animals (Penaflorida, 1955). Additionally, Venkatramalingam et al. (2017) studying the tiger prawn *(P. Monodon spp*) reported that when the post-larvae were fed with the herbal appetizer *Zingiber officinalis* enriched *Artemia,* animals have their digestive enzymes improved (amylase, protease, and lipase), resulting in higher weight gain and SGR, and better FCR.

It has been established herbs and herbal extracts also, present anti-inflammatory effects that could have contributed to the satisfactory growth and health parameters observed in the present investigation. As revealed by Churchid et al. (2017), the anti-inflammatory action of the polyphenols presented on the grape pomace increased the average body weight of *L. vannamei* post-larvae. In particular the authors noticed that animals fed separately with a yeast cell wall did not present the same growth-improving effect as that animals fed with the grape pomace alone. In our study, we tested both yeast cell wall and terrestrial herb together as a blend, and we emphasize the relevance of the natural blend administered as a composition in order to lead the observed

results. Animal performance were statistically superior in animals fed with the immunonutritional blend, when compared to control group. Likewise, shrimp gut microbiota was positively modulated by the dietary inclusion of yeast cell wall and terrestrial herb, with the preservation of a healthy gut microbiota.

Remarkably, the main reason for the use of immunostimulants in aquaculture is to attain optimal production, through achieving growth stimulation and promoting animal health and survival. In order to estimate the influence of YAH on shrimp farming output, we measured the final shrimp yield. Indeed, the addition of YAH resulted in improved final yield, demonstrating that the inclusion of yeast cell wall and herbs in *L. vannamei* diet enhanced this indicator and would contribute to a better income and profit and to the overall improvement.

In fact, although the β-glucan has no specific immunostimulatory effect (i.e., it enhances the immune system without antigenic specificity) (Ganguly et al., 2010), it presents a strong immunostimulating activity which, along with the several botanical compounds (e.g. polysaccharides, polypeptides, organic acids, alkaloids, glycosides, among others), associated with herbal extracts. This synergetic and complementary effects may have led to the results observed in this study. Certainly, the continuous shrimp feeding management with the synergistic blend not only showed no side effects on animal performance but also improved the zootechnical parameters, particularly animals that received 1% of YAH dietary inclusion.

Furthermore, it is essential that continuous monitoring of the shrimp immune health occurs. Substances, such as immunostimulants, can be recognized as a “foreign” element by the shrimp immune system if used repeatedly, resulting, therefore sufficient control must be applied to any functional feed supplement to obtain maximum performance an undesirable immune-fatigue with the depletion of the defence system (Smith et al., 2014).

When analysing the inclusion of a new component to aquafeed, it is imperative that it does not lead to an intestinal dysbiosis (i.e. a microbial imbalance) under any circumstances. Furthermore, if the new compound favours a beneficial bacteria selection, the greater is its advantage a healthy shrimp farming. Based on our results, it is possible to affirm that the dietary YAH inclusion for juvenile *L. vannamei* did not lead to any adverse intestinal microbiota imbalance. Even more, the blend of immunostimulants also contributed to the increase of a relevant probiotic genus, among other favourable influences.

The addition of 1% YAH was the treatment that most influenced the shrimp intestinal microbiota composition. This finding was also corroborated with the PCoA results, that revealed a distinct separation and dissimilarity between 1% YAH treatment and control group fed shrimp. Animals that received this percentage of immunostimulant presented the phylum Proteobacteria as the most prevalent. This phylum, in fact, has been reported by other studies as the most dominant in shrimp (Xiong et al., 2017; Li et al., 2018). Meanwhile, the Fusobacteria phylum was the most prevalent in the digestive tract among the animals that received 0.5% YAH and those from the control

group. This phylum has also been described as one of the most prevalent phyla in the intestine of the Pacific white shrimp. *De facto*, these two phyla, among others, were described to be part of the autochthone gut microbiota of *L. vannamei*, since larval until adult stage (Zeng et al., 2017). Luis-Villaseñor et al. (2013) showed that the phyla Proteobacteria, Fusobacteria, Sphingobacteria, and Flavobacteria were the most prevalent in the gut of *L. vannamei* after being fed with a *Bacillus* probiotic mix. Moreover, these two phyla were reported to be upregulated by WSSV infection (Wang et al., 2019b).

Those findings can also be observed in the most dominant microbial generain this experiment. The genus *Cetobacterium* was the most predominant in all treatments, although significantly lower in 1% YAH. This genus, and specifically *C. somerae* species, is related to the production of vitamin B_{12} (cobalamin) in fish (Rodiles et al., 2018; Tsuchiya et al., 2008). On Chinese crabs, cobalamin is associated with the nonspecific immune responses (Wei et al., 2014). In shrimp, vitamin B_{12} is commonly supplemented as a form of cyanocobalamin, in optimal doses of 0.1 – 0.2 mg/kg in complete diets (Koshio, 2014) Although there is recent research showing the a relationship between *Cetobacterium* and cobalamin in fish, there is a lack of studies on this bacterium and its role in shrimp. As the most prevalent genera in the three analysed group of the present study, certainly this genus is worthy of future research.

Similarly, the genus *Sphingobium* was higher in the 1% YAH group in comparison to the control group, indeed reaching a relevant percentage of relative abundance for that treatment. Sphingobium was isolated from fresh water and filteredwater (Sheu; Shiau;

Cheu, 2013; Corre et al. 2019) and, as some bacteria from this genus may degrade polycyclic aromatic hydrocarbons, they can be used for soil bioremediation (Chen et al., 2016).

Moreover, in the LEfSe analysis, this genus was enriched and presented the biggest LDA score, showing that this genus had great relevance, and could explain part of differences between this treatment and the other groups. It is a relatively new described genus, first proposed by Takeuchi et al. (2001) and, at the moment, it is only mentioned to be part of some intestinal shrimp microbiota (Hu et al., 2017), but its relevance is rarely discussed. The genus counts as the 40 most common taxa found in the arthropod gut microbiota, from soil and the aquatic environment (Esposti; Romero, 2017), was isolate from the rhizosphere of an aquaponics system (Schmautz et al., 2017) and associated with an antibiotic resistance (glycopeptide resistance gene) in an experimental aquaculture facility (Colombo et al., 2016). The role of this genus on the gut microbiota of shrimp from the present study remains unclear.

Noteworthy, the inclusion of YAH on shrimp diet, both 0.5%, and 1.0%, significantly increased the relative abundance of the genus *Bacillus* in the gut microbiota. This result is remarkable due to the significant probiotic importance of this genus. *Bacillus* is considerated as a autochthonous member the of crustacean's environment and is among the widely used probiotic bacteria for crustaceans (Castex; Daniels; Chim, 2014), mainly due to its capacity to activate both cellular and humoral shrimp immune responses (Rengpipat et al., 2000) and to its naturally produce antibiotic compounds (Van Hai; Fotedar, 2009). Thus, the *Bacillus* stimulation by the YAH diet inclusion is a favourable influence towards the maintenance of a healthy shrimp gut microbiota.

As an added bonus, the inclusion of YAH led to almost no OTU suppression. In fact, only 1.9% of the identified OTUs were only found in the control group, and we may infer that the inclusion of the YAH into the shrimp diet did not suppress an excesive number of microorganisms. On the contrary, one-third of the OTUs were found exclusively within the gut microbiota of animals that received the YAH, including *Exiguobacterium* and *Vibrio. Exiguobacterium* has been proposed to be a potential probiotic for *L. vannamei* (Cong et al., 2017) and may increase shrimp survival and growth (Sombatjinda et al., 2014). Moreover, this genus has potential biotechnological use to industry and agriculture as a plant growth- promoting (Kasana; Pandey, 2016). Equally, although *Vibrio* genus also encompasses some opportunistic pathogens, others may act as probiotics for crustaceans (Castex; Daniels; Chim, 2014), such as *V. alginolyticus* (Austin et al., 1995). Further, *Vibrio* may play a relevant role in shrimp nutrition digestion, due to its genes related to digestive enzymes (Gao et al., 2019).

Lastly, even with changes observed in the gut microbiota composition due to the addition of YAH especially on 1% treatment that was dissimilarity to control group, we noticed a stable core microbiota. A stable and permanent bacterial community was preserved, regardless the addition of YAH in the shrimp aquafeed. The core microbiota, composed by LAB (lactic acid bacteria) and recognizable or promising probiotic strains in this study, is intimately associated with healthy and diseased animals, being paramount on the host-bacteria interaction. A healthy shrimp gut microbiota is characterized by a high diversity with cooperative interactions, while diseased animals tend to have less diversity and a simpler gut microbiota (Yao et al., 2018). Moreover,

when elucidating the core microbiota composition, it is easier to manipulate it in order to develop effective strategies to promote animal health and growth (Steinberg, 2018). Thus, the preservation of a healthy and balanced gut microbiota, as observed in the inclusion of YAH, may result in more resilient and stronger shrimp, likely to better respond to stressful situations.

In conclusion, the present study has implied that the inclusion of an immunostimulant consisting of a yeast cell wall and terrestrial herbs in diets for juvenile *L. vannamei* raised on an intensive system and under similar commercial culture pond conditions resulted in improved shrimp farming conditions. Due to its dietary influences, YAH can be supplied to shrimp as a quantifiable prophylactic agent in order to promote animal performance and gut health. Moreover, as the product is “green” and adds value to shrimp production, it may contribute to the final product quality and standards of shrimp production this obviously has advantages to the producer.

4.6 Conclusions

1. YAH dietary inclusion increases survival and results in better feed conversion ratio (FCR) for *Litopenaeus vannamei.* The 1% YAH inclusion level led to superior weight gain and growth rate (SGR).

2. The immunostimulant tested affects shrimp performance partially due to beneficial gut microbiota modulation and likely metabolic improvements

3. Our results support the evidence that 1% YAH inclusion level modulates shrimp gut microbiota by significantly changing Phylum and Genus level and additionally do not appreciating alter core microbiota;

4. Several beneficial bacteria such as are promoted by the YAH dietary inclusion. Those beneficial strains may act a “bio-friendly" agent improving shrimp defences, systems allowing enhancement stress and disease resistance

5. YAH can be used as prophylactic agent in shrimp farming without deficit of productivity or loss in animal health this reduces current dependency on chemotherapents and medicines in shrimp farm management during high risk and disease episodes.

CHAPTER 5: Modulation and gut microbial ecology using a commercial Tuna Liquid Hydrolizate (TLH) in diets for shrimps (*Litopenaeus vannamei*)

5.1 Introduction

5.1.1 The role of tuna fisheries and hydrolysates in Mexico

In the global fisheries industry, tuna in of Mexico's most relevant activity with two main species dominating the market, yellow fin tail and blue fin tuna for high quality markets such as Japan. However, during the tuna canning process, about ~ 52 - 54 % of the total fish weight is discarded as waste (Hernández et al. 2013). Instead of being wasted, the residual product can be used to obtain tuna by-products and Tuna Liquid Hydrolysates (TLH). Obtained from tuna liver and other wastes, TLH is a promising alternative feed ingredient that can be promoted as a functional dietary supplement, in addition it adds value to the residues resulting from the industrialization process of tuna. TLH can be used in aquaculture food industry to provide functional effects such as attractant and texture properties. Furthermore, TLH is a potential source of antioxidant peptides such as the protein from bigeye tuna and yellow fin tuna (*Thunnus obesus, Thunnus albacares spp*) backbone protein and dark muscle, which can enhance shelf life product as well as improve final product stability (Je et al. 2008). Other properties of TLH are antianemia compounds and components to be used in microbial growth media (Herpandi, Rosma, Nadiah 2011). In addition, Ahn et al. (2010) also reported functional peptides from tuna liver.

The hydrolysis reaction is a cleavage with the addition of water, causing depolymerization of proteins, carbohydrates, and nucleic acids (Nelson; Cox; 2005).

Protein hydrolysate is a protein that has been cleaved into small or large polipeptides, peptides, presenting nutritional and physiological functions in animals such as livestock, poultry, swine and fish. Moreover, protein hydrolysate also has antimicrobial, antioxidant, antihypertensive, and immunomodulatory activities (Hou et al., 2017). TLH, obtained by enzymatic hydrolysis, has been described presenting texturing properties, anti-anemia compounds, and a promising source of antioxidant (Je et al., 2008; Herpandi, Rosma, Nadiah 2011). Furthermore, TLH may confer biological functions with functional and bioactive peptides (Ahn et al., 2010).

The protein hydrolysates, obtained by enzymatic hydrolysis using various proteases, showed to be a value-added product, with excellent antioxidant activities, which can be widely applied to improve and upgrade the functional and nutritional properties of proteins. Therefore, a complete amino acid profile can be an excellent nutrition option for juvenile and grownt out shrimps, with health benefits and with considerable potential of replacing fish meal by TLH (Hernández et al. 2004).

5.1.2 Environment and sustainability

TLH presents itself as a sustainable and alternative ingredient in comparision to traditional one, due to not only its lower price but also because of its relatively consistent composition and supply. On the other hand, plant proteins, such as oilseed cakes are often economically and nutritionally valuable sources of protein; however,they have potential problems associated with insufficient levels of essential amino acids (particularly lysine and methionine) and contain antinutritional factors (ANF's).

Presently, feed manufacturers face increasing prices of almost all imported feed supplies. As aconsequence, efforts have been made to study the feasibility of utilizing potential local ingredients; however, their use will depend on sufficient primary material supply and cost. In addition, it will be necessary the ability to commercially compete with human food supplements, such as agricultural by-products, oil crops, by-products (Hardy 2010) and fisheries by-products (Hernandez et al., 2004). Figure 5.1 shows the Peruvian fishmeal price evolution in 30 years, per metric ton.

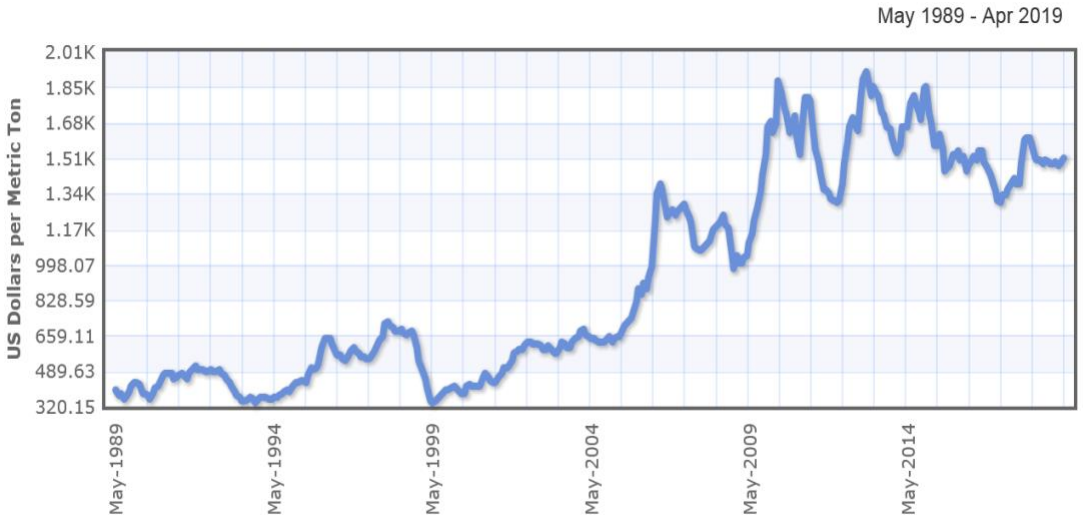


Figure 5.1: Peruvian fishmeal 65% protein. US Dollars per metric ton. Source: World Bank Commodity Price. Available on: www.indexmundi.com/commodities

5.1.3 Feed palatability and shrimps

For shrimps, feed palatability is closely related to the presence of attractants compounds, normally associated with the shrimp´s prey components under wild conditions. Thus, nutritional and sensorial acceptable diets are essential to achieving satisfactory intake and performance. For crustaceans, feeds components with high attractability are those with low molecular weight compounds, soluble in water and

ethanol, and related to potential prey items . These compounds include free amino acids, especially taurine, hydroxyproline, glycine, organic acids, nucleotides, and nucleosides, and small peptides stimulate shrimp feeding (Tantikitti et al 2013).

Regarding the economic aspect, the feed attractability and palatability is crucial to shrimp performance in aquaculture. Shrimp may be selective (Suresh; Varagam; Nates, 2011), thus to achieve satisfactory intakes, resulting in a successful shrimp farming, it is necessary that palatability studies newly emerge, and the economic impact of the new feed additive be correctly measured. Figure 5.2 illustrates the relationship between feed palatability and good farm management.

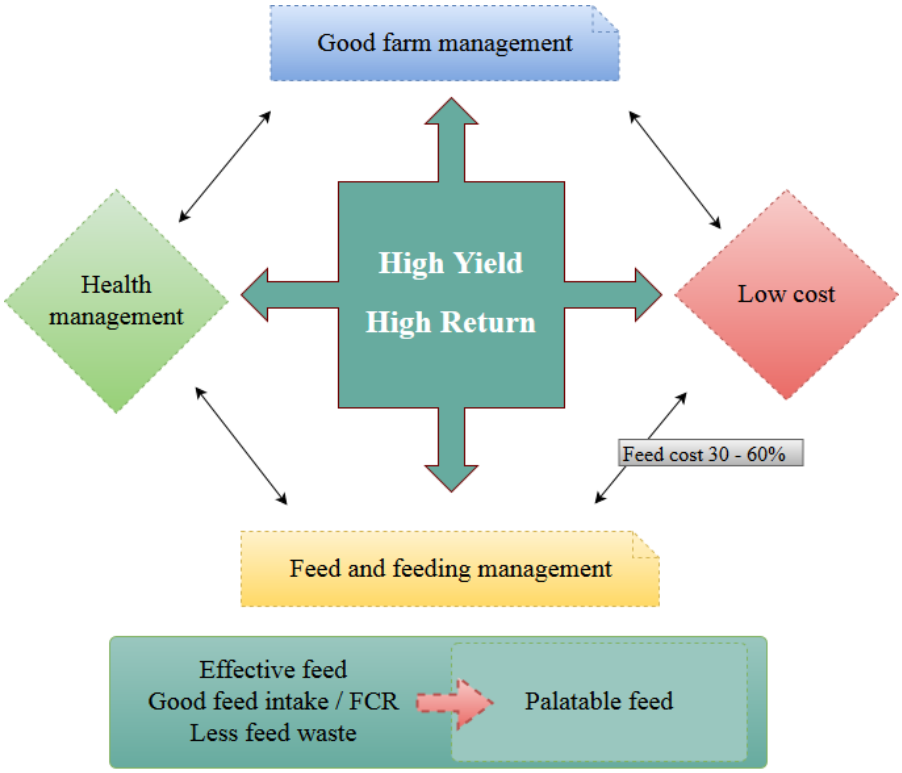


Figure 5.2: Key factors in aquaculture production, showing the importance of feed palatability. Source: Tantikitti, 2014.

The complex peptide profiles for tuna hydrolysates are very interesting from the above standpoint and therefore their functional properties are worthwhile evaluating the shrimp. Their potential to modify the gut microbiota in fish and shrimp should be further explored.

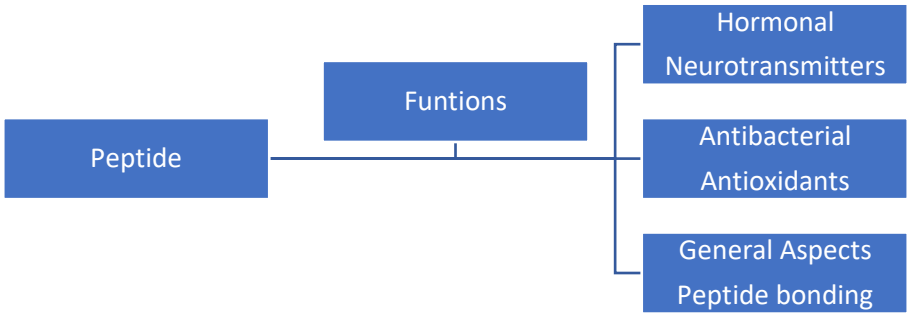


Figure 5.3: Summary of the main functions of the peptides in crustaceans.

The use of appropriate techniques to understand the modelution in bacterial communities such as clone library analysis, denaturing gradient gel electrophoresis (DGGE), and High Throughput Sequencing (HTS) may facilitate a deep understanding of the gut microbiota (Liu et al., 2011; Huang et a., 2016).

Despite the efforts to study the shrimp gut microbiota, questions about the role of novel feed additives, such as hydrolysates from tuna on gut function and their effect on remodeling the gut microbiota and the nonspecific immune response arise. Therefore, the main purpose of the present research trial was to assess the effect of TLH on shrimp performance and gut health, in terms of growth performance, feed

acceptance, and gut microbiota modulation and morphology using animals raised in an intensive commercial system. The inclusion of TLH in shrimp diets has been reported previously to have significant impact on the reduction of colonization of pathogenetic bacteria in the gut (Nguyen et al. 2012). It has been stated that the use of TLH can modify the gut structure in numerous species with the inclusion of TLH (Siddik et al. 2018).

5.2 Purpose and objectives

The purpose and objectives of the investigation study was to identify the potential environmental and microecological aspects of the gut microbiota in cultured shrimp and their health status. To achieve the objectives, quantified decrease and increase of taxa presence in comparison with indigenous microbial community and biological relevance was studied. A 105-day feeding trial with floating cages inside an intensive shrimp pond was performed to obtain zootechnical parameters and shrimp samples for HTS, evaluating at the inclusion of a commercial tuna liquid hydrolizate (TLH) at two inclusion levels to reduce any pathogenic bacteria in the posterior intestine of *Litopenaus vannamei.*

1. To estimate the influence of shrimp perfomance and survival with the use of TLH.
2. To evaluate the modulation of bacteria phyla and genus when fed with TLH
3. To identify the core microbiota and the exclusive OTUs with the use of TLH.

5.3 Materials and methods

5.3.1 Experimental design

The trials described in this research were conducted at a small commercial shrimp farm located in Tecoman, Colima, Mexico (18.9174 °N, 103.8738 °W). The region is well known for high-density shrimp farming, with over four to six paddle wheels (2hp/unit) as aerators with a capacity of 8-12 hp/pond within 0.5-hectare ponds with 2.5 meters deep with central drainage to keep high densities with low organic and suspended solids in where floating cages were placed and used for experimental trials with same water culture conditions as other commercial ponds.

Figure 5.4: Location of the intensive shrimp farm unit named “Granja Acuícola Los Tucanes”, Tecoman city, Colima State, Mexico, where the trials described in this work occurred. (Google maps@2016 Google images, Access: 09. Apr. 2018).

As mentioned in previous chapters cages were randomly located in an intensive pond with 65 PL/m^3 with 3.00 ± 0.25 g size shrimp coming from nursery phase. Feeding

regime was adjusted based on density and biomass, with the use of feeding trays and the farm feeding protocol simulating commercial and adjusting feed was sumistrated twice a day, its worthy to mention the cages had no interaction with the earth bottom pond.

5.3.2 Experimental animals and housing

All experimental shrimps were kept in a nursery of 100 m^3 for 22 days prior stocking in the ponds (0.001 g to 0.500 g), once transferred in the pond sixteen cages were placed anchored, same protocol was used to select shrimp from ponds as mentioned on chapter 2 the ideal size for cages and net mesh 3.00 g ± 0.25 g size. Once shrimp was located into the cages regular monitoring occurred with maintanece, revisions and feeding rate according to farms table.

In general all parameters where under regular parameters scepter the feed consumsion as seen on in the results, on week 3-4 our feeding strategy was changed due to personal observation when delivering the feed, first feeding trays where clean after 30 minutes (normally is 60 to 90 minutes in this particular site), secondly temperature rose up to 33 ◦C in where physiological activities increases and lastly the oxygen increase was a favorable parameter to allow digestibility and growth.

Therefore, I took the decision to increase to feed intake dramastically and see if we could gain weight faster and challenge the animals, also regular monitoring show little mortalities.

5.3.3 Experimental diets and ingredients

Three experimental shrimp diets were tested, i.e., two commercial TLH (*Tuna liver from Tunnus albacares spp)* dietary inclusion were evaluated, specifically 2% and 4% TLH inclusion, against the control diet (0% inclusion) (Table 5.1). A twenty-five percent feed formulation was used for his trials, the protein ingredients were lower in protein quality and quantity, as part of the trial and the natural characteristics of TLH from promoting feed intake and performance, also a base line to compared with previous trials from this experimental work. Unfortunately, these feed formulations were not made for high density farming as total 25 % CP shrimp diet could have affected results due to the site conditions and intensification, nevertheless the parameters observed were similar to standars yield in this geographic location.

Feed was manufactured by Nutrimentos Acuicolas Azteca SA DE CV, producing batches of 5000 kg at 1.5 -2 mm size pellet. Tuna liquid hydrolysate (Grupo Diana Pet food, Mexico) was added in the feed mixture as part of the shrimp diet formulation, all diets were isoproteic and isoenergetic.

Ingredients (g.kg^{-1})	Control	2 %	4 %
Fish meal	6	6	6
Soybean meal	27	27	27
Poultry meal	16	16	16
Wheat flour	43	43	43
Tuna Hidrolizate (TLH)	0.0	0.2	0.4
Lecithin	1	1	1
Fish oil	3	3	3
Soybean oil	1	1	1
Vitamin premix b	1	1	1
Mineral premix c	1	1	1
Vitamin C	0.3	0.3	0.3
Antifungal	0.1	0.1	0.1
Analysis (g.kg^{-1}; dry weight basis)			
Moisture (%)	6	6	6
Crude protein (%)	25	25	25
Crude lipid (%)	8	8	8
Ash (%)	15	15	15

- 56/7 Tuna by product Fish Meal, Colima, Mexico.
- 55/3 Soybean meal, USA
- 62/6 Poultry meal, Tyson
- 7/2 Weat flour, Mexico
- Lecitin, Mexico
- Fish oil, Aqua- Chile
- Soybean oils, USA
- Vitamin premix b, Vimifos
- Mineral premix c, Vimifos
- Vitamin C, Vimifos
- Antifungal, Vimifos
- Vimifos- Mexico.

Table 5.1. Feed formulations and proximate composition of the experimental diets for shrimp in the trial.

5.3.4 Water quality parameters

Water quality parameters were monitored during all the trial course, and all of them remained within the appropriated level for shrimp farming. Average water temperature was 29.66°C ± 0.82 in the morning and 32.17°C ± 0.56 in the afternoon (Fig. 5.5), and average dissolved oxygen was 2.87 $mg.L^{-1}$ ± in the morning and 4.58 $mg.L^{-1}$ ± 0.77 in the afternoon (Fig. 5.6). Other parameters are presented in Table 5.2.

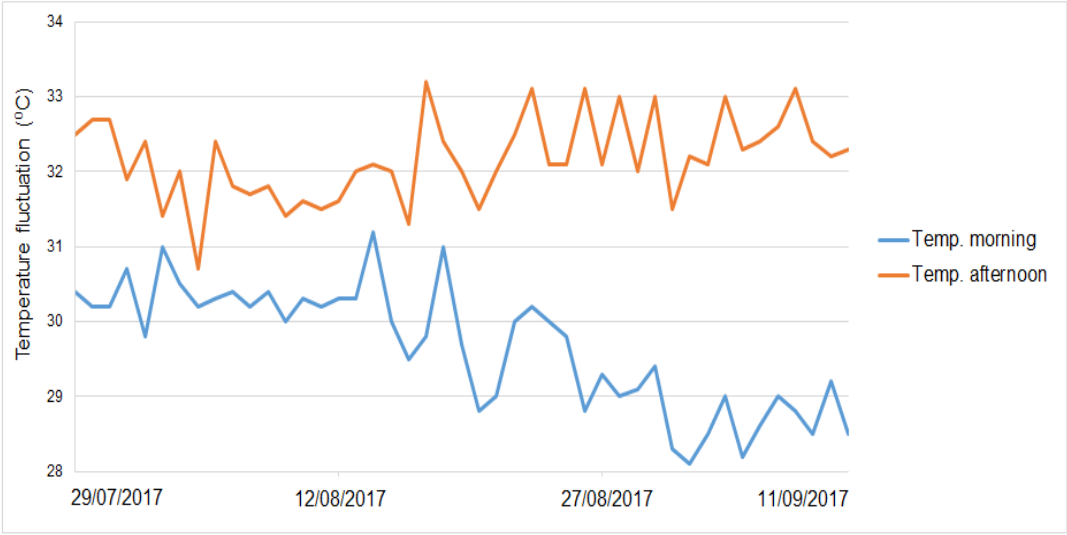
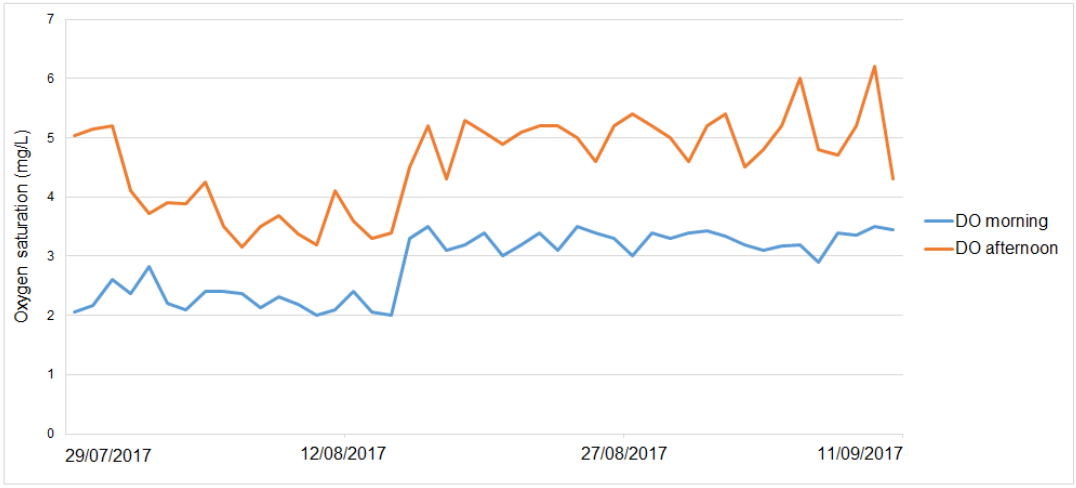


Figure 5.5: Water temperature fluctuation during the trial, day and night measurement.



DO: Dissolved Oxygen

Figure 5.6: Oxygen saturation during the trial, day and night measurement.

Table 5.2: Water parameters, salinity, pH, Ammonia and nitrates.

Parameters	Results
Total salinity (ppt)	4-5
pH	8.28 – 8.96
Ammonia ($mg.L^{-1}$)	>0.025
Nitrite ($mg.L^{-1}$)	> 0.001

5.3.5 Performance and biomass sampling

Throughput the course of the feeding trial, tank water quality monitoring, feed consumption, and mortalities were recorded daily. Feed adjustment, growth, and health characteristics were recorded and observed every week by weighing a pooled sample of the population (n= 40 PL per cage, i.e., thirty percent of the population per cage), using a DS Scale. This monitoring allowed the calculation of growth performance, zootechnical parameters, FCR (feed conversion ratio), and an inferred economic analysis based on survivals, biomass produced and FCR in where correlated farm indicator where compared.

After eight weeks, the cages were harvested with the help of farm staff and students in order to synchronize sampling work, i.e., weighing, data recording, dissection, sample fixation, and labeling. Shrimp were individually weighted, counted, and collected for sampling. Animals were euthanised by thermal shock (~ 33 °C to 9 °C) and their surface were cleaned with 70% (v/v) ethanol. Posterior gut with its content and hepatopancreas were removed with sterilized tweezers and scissors and fixed in 70% (v/v) molecular ethanol, being stored in sterile 2 ml microtubes and kept in -18 °C for conservation. Samples were shipped on dry ice to the University of Plymouth, UK, under the appropriated importation license in the United Kingdom Customs (Home Office Licence).

5.3.6 Peptide profile analysis

With the use of novel analytical methods of exclusion as chromatography (HPLC), we analyzed the peptide molecular weight profile of the experimental shrimp feeds with the inclusion of TLH. This method provides a "fingerprint" of the feed ingredients (Lian; Lee; Park, 2005; Stranska-Zachariasova et al., 2016), being an extensive analytical technique, which outcome can result in several benefits, such as product development, quality control, competition analysis, marine raw materials analysis, and aquafeed analysis (Altunok et al., 2016; Habibi et al., 2017; Nolvachai; Kulsing; Marriott, 2017).

A Resin column (200- 15000 Da), was used to obtain molecular weight classes, peptides and protein, calibration curve with 7 standard peptides, reading absorbance at 214 nm, % of peptides calculated by interacting area under the HPLC curve.

Table 5.3: Relative profile soluble peptides in experimental diets.

Relative profile (%soluble pep)	AZTECA C	AZTECA 2	AZTECA 4
	13/10/17	13/10/17	13/10/17
	Ech 1	Ech 2	Ech 3
MW>20 000	1.24	0.89	1.21
10 000 – 20 000 Da	4.62	4.17	4.75
5 000 - 10 000 Da	7.15	7.52	7.48
1 000 - 5 000 Da	10.80	13.68	12.46
500 - 1 000 Da	5.02	5.64	5.31
500 > MW	71.18	68.10	68.77
Total	100.0	100.0	100.0

MW: Molecular weight.

5.3.7 Sample collection

A number of samples were collected from cages and treatments to perform DNA extraction and sequencing thru Ion Torrent and analyzed under QIIME database and to see bacterial communities' abundancies and presence. In the search to find correlations between bacterial found in the shrimp gut, feed additive inclusion and environment and pond conditions. We develop a map from samples and treatments to actually see where each shrimp was kept during the feeding trial (Figure 5.9), in addition to Tables 5.3 and 5.4 that shows origin of each sample.

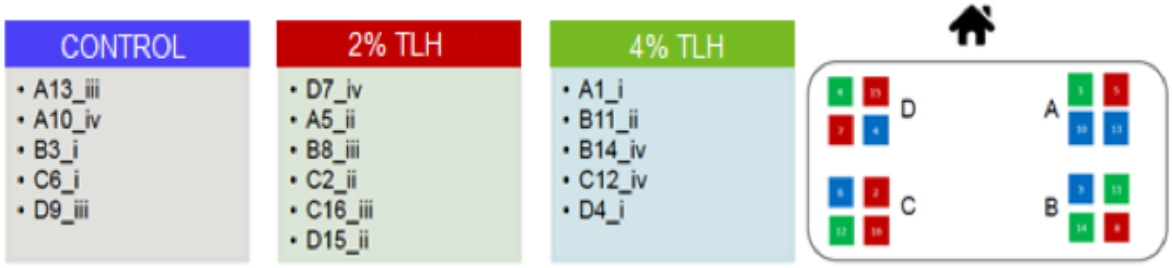


Figure 5.7. Map of samples and l location from cages in the pond.

Table 5.3 Treatments and colour ID

Treatment	ID Colour	TLH inclusion [%]	Replicates
Control Treatment	Red	0%	5
Control Treatment	Green	2%	6
Control Treatment	Blue	4%	5

Table 5.4: Samples origin from cages and Ion Torrent code

SAMPLE #	Cage CODE	Ion Xpres	Treatment CODE	Sample
11	A12 1	1	Blue	30 µl
12	A4, 1	2	Blue	30 µl
17	A14,2	7	Blue	30 µl
21	A14,3	11	Blue	30 µl
30	A4, 3	17	Blue	25 µl
31	A12, 2	18	Blue	25 µl
13	A1,3	3	Green	30 µl
15	V5,4	5	Green	30 µl
16	V8,3	6	Green	30 µl
20	V2,2	10	Green	30 µl
25	V7, 3	15	Green	25 µl
26	V16, 3	16	Green	25 µl
14	V2,4	4	Red	30 µl
18	R6,2	8	Red	30 µl
19	R3,1	9	Red	30 µl
22	R3, 4	12	Red	25 µl
23	R13, 1	13	Red	25 µl
24	R13, 4	14	Red	25 µl

5.3.8 DNA extraction

The molecular analysis from the shrimp gut microbiota was performed at the Microbiology laboratory at the University of Plymouth, UK, where I performed DNA extraction wit the support of my collegues. In where 16S rRNA gene was amplified through PCR (polymerase chain reaction). Subsequently, the amplified and purified DNA was sequenced with in the Systems Biology Centre at University of Plymouth.

Bacterial DNA extraction was carried out using the QIAamp Stool Mini Kit (Qiagen®), following manufacturer's guidance and instructions. A first step was added, i.e., an initial incubation with 50 mg/ml of lysozyme for 30min at 37 ◦C, in order to potentiate the lysis of Gram-positive bacteria. DNA was extracted from 18 intestinal samples (n=6 per treatment) (13.88 mg ± 2.57 mg) of *Litopenaeus vannamei*. Purity and quantity of

the extracted DNA were evaluated using a UV spectrophotometer (NanoDrop™ 2000 Spectrophotometer, ThermoFischer Scientific®), measuring the absorbance in 260/280 nm and 260/230 nm.

5.3.9 16s r RNA sequencing

V1-V2 region of the prokaryotic 16S ribosomal RNA gene was amplified by polymerase chain reaction (PCR) assay, using the primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and a pool of primers 338R-I (5'GCW GCC TCC CGT AGG AGT 3') and 338R-II (5' GCW GCC ACC CGT AGG TCT 3'), designed by Gajardo et al. 2016, to amplify a fragment of 350 bp. *Touchdown*-PCR was carried out with 25 µl of MyTaq™ Red Mix (Bioline®), 1 µl of each primer (25 pM), 1 µl of DNA template (1 ng/µl), and ultrapure DNase free water in a final volume of 50 µl. Table 3 presents the amplification cycling profile. In each amplification reaction, DNA of *Escherichia coli* was used as positive control and, as a negative control, ultrapure water. Amplified products were confirmed by electrophoresis in 1.5% agarose gel, with SYBR Safe DNA gel stain (ThermoFischer Scientific®), in TAE buffer at constant voltage (80 V) for approximately 40 minute and visualized under UV light.

Table 5.5: Cycling profile of the t*ouchdown*-PCR amplification

Phase	Temperature (°C)	Time	Cycles
Initial denaturation	94	7’	
Denaturation	94	30”	
Hybridization	63 - 53	30”	10 x touchdown
Elongation	72	30”	
Denaturation	94	30”	
Hybridization	53	30”	25 x
Elongation	72	30”	
Final Elongation	72	10’	
Finalization	10	Until end	

Pooled PCR products were purified using the magnetic beads technology, with AMPure XP (Beckman Coulter®). After bead purification, the amplified and purified DNA was addressed to Systems Biology Centre of University of Plymouth UK, Genomics Facilities, for next-generation DNA sequencing, employing Life Technologies Ion Torrent™ Personal Genome Machine™ System (ThermoScientific®).

5.3.10 High Throughput Sequencing

Raw Sequence data were trimmed from low-quality scores (Q score < 20), with FASTX-Toolkit (Hannon Lab). Data were then analysed using Quantitative Insights into Microbial Ecology (Qiime 1.8.0) (Gajardo et al., 2016). Operational Taxonomic Units (OTUs) were sorted and filtered with 97% of sequence identity. RDP (Ribosomal Database Project) tool was used to ascertain taxonomic affiliation, with 0.8 of confidence. Alpha and *β* diversity were calculated with ape, vegan, and R. Bacterial richness and diversity were determined with α rarefaction, by Chao1, Observed species, and Phylogenetic Diversity. Good´s coverage was also identified. Similarities and diversity were estimated with β rarefaction. Weighted and Unweighted UniFrac distances were calculated, and similarities and dissimilarities between treatments were

investigated by Principal Coordinate Analysis (PCoA). Phylum and genus taxonomic rank were presented as relative abundance graphs. LEfSe (Linear discriminant analysis effect size) tool was used to determine biomarkers or differential taxa between treatments, using relative abundance on the OTUs tables (Segata et al., 2012), with alpha significance of 0.05 and effect size threshold of 2. Core microbiota was identified using Venn diagrams, through Venny 2.1 software (http://bioinfogp.cnb.csic.es/tools/venny/, Oliveros 2007-2015). Data is presented as mean ± SD. p-value < 0.05 was considered statistically significant.

5.4 Results

5.4.1. Zootechnical performance

Regarding shrimp survival, there was no significantly different among experimental groups. Despite this, animals with 2% TLH diet presented almost 1% better survival than the control group. Control group and 2% TLH showed a survival above 92%, while treatment with 4% TLH displayed 88% of survival. It is worthy to notice that all treatments maintained an excellent survival, always above commercial standards. Survival (%) along the experiment is presented in Figure 5.10.

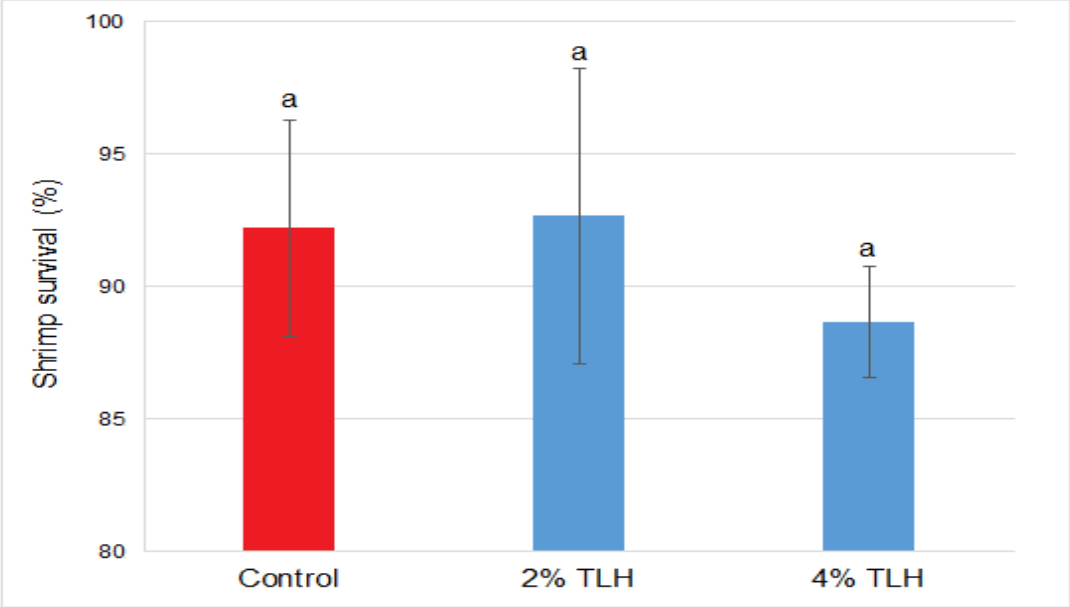


Figure 5.8: Survival of *Litopenaeus vannamei* with two different Tuna Liquid Hydrolysate (TLH) diet inclusion and control group. Similar letter means no significant differences (ANOVA, p=0.3125). Data are average

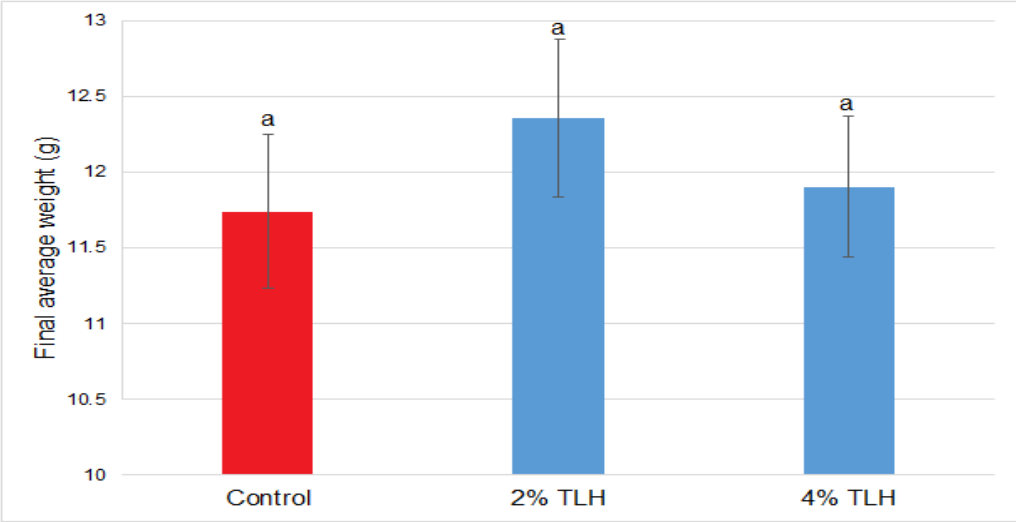


Figure 5.9: *Litopenaeus vannamei* final weight gain with two different Tuna Liquid Hydrolysate (TLH) diet inclusion and control group. Similar letter means no significant differences (ANOVA, p=0.9822). Data are average.

In relation to shrimp weight gain and growth, there was no statistical difference between treatments among these two ratio. However, it is relevant no notice that the 2% TLH group presented an improvement of 9% in growth, and the 4% TLH group showed a 4% improvement in the metric, in comparison to control group. Figures 5.11 and 5.12 illustrate the shrimp final weight gain and specific growth rate, respectively.

*Data were calculated with the average per group.

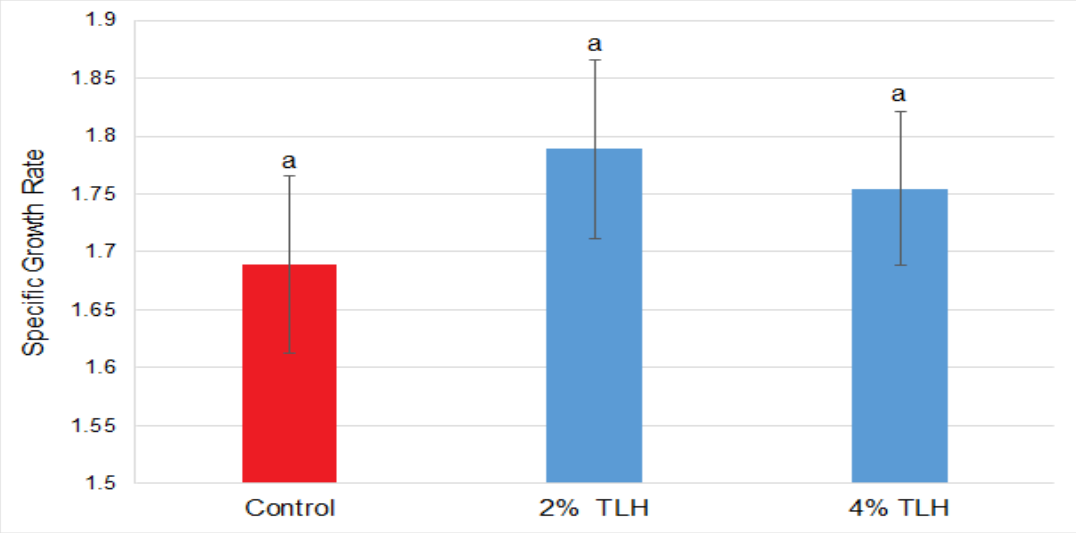


Figure 5.10: *Litopenaeus vannamei* specific growth rate with two different Tuna Liquid Hydrolysate (TLH) diet inclusion and control group. Data were calculated with the average per group ± SD. Similar letter means no significant differences (ANOVA, p=0.1349).

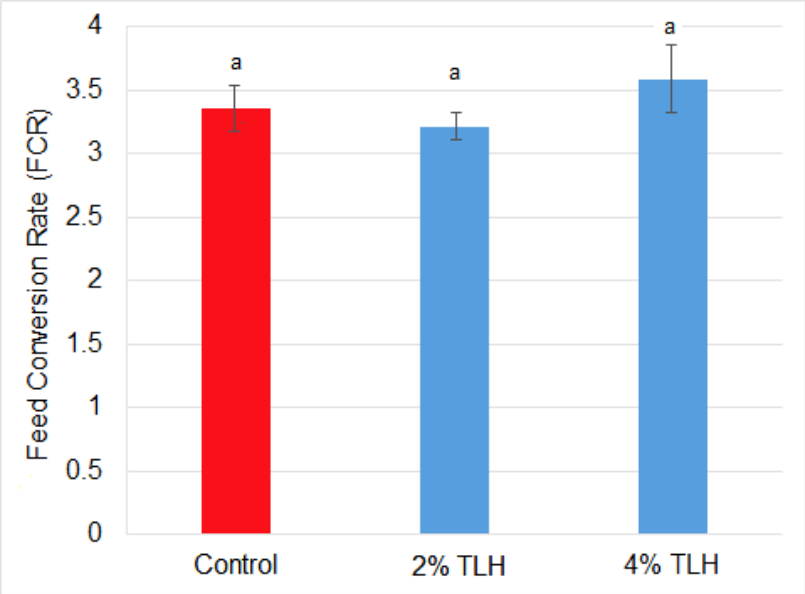


Figure 5.11: Shrimp gross Feed Conversion Rate (FCR) with two different Tuna Liquid Hydrolysate (TLH) diet inclusion and control group. Data were calculated with the average per group. Similar letter means no significant differences (ANOVA, p=0.0813).

Regarding shrimp feed conversion rate (FCR), no significant differences were found between treatments. Diets with 2% TLH inclusion and control group showed the best FCR, while treatment with 4% TLH dietary inclusion resulted in a higher FCR, but not significant. Although FCR of the 2% TLH treatment was shown to be 4% lower than the control group, this difference was not statistically significant. In general, the high values of FCR observed in this study can be understood due to the high feeding rate based on feed-intake observed in feed trials. This situation could be avoided following the indicators of the feeding tables as a function of the average weight and percentages of corresponding biomass. Figure 5.13 shows the shrimp feed conversion rate.

Concerning a predicted cost benefit economic analysis, an extrapolated net production and a possible return of investment were calculated (Fig. 5.14 and Fig. 5.15). The inferred net production showed no significant differences between the three groups,

although the 2% TLH inclusion treatment presented the best result of all, showing an additional of 10% in terms of productivity against the control group. In addition, an analysis of a possible return of investment was calculated, based on standardized values of price, fixed cost, and cost of feeding and juveniles. These values were used to calculate the total cost and the net benefit for treatment. This analysis revealed a strong convenience to include 2% of TLH in *L. vannamei* diet, which would increase the return on investment by 6% in comparison to 0% inclusion diet (p=0.0234).

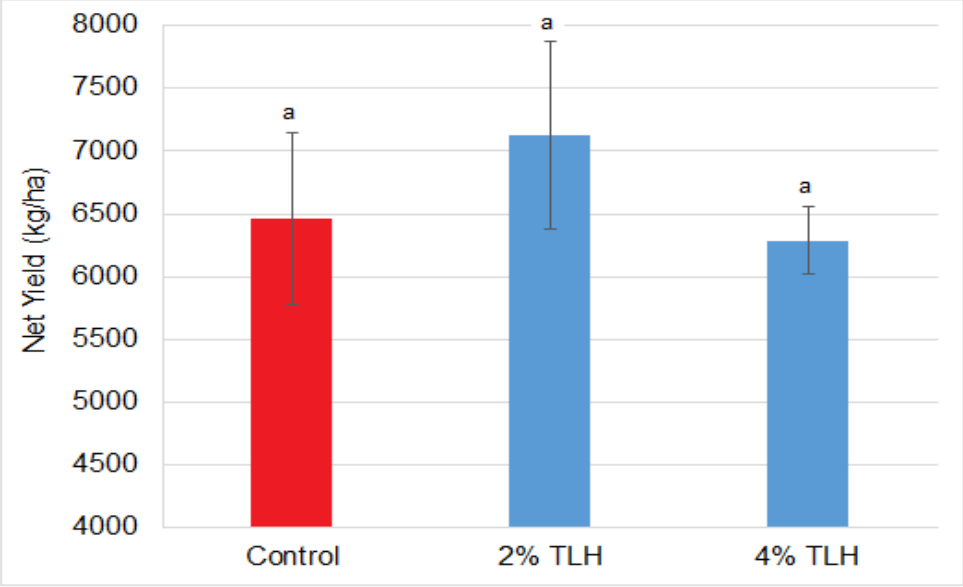


Figure 5.12. Extrapolated net production of two different Tuna Liquid Hydrolysate (TLH) diet inclusion and control group, for *Litopenaeus vannamei*. Data were calculated with the average per group. Similar letter means no significant differences (ANOVA, p=0.1655).

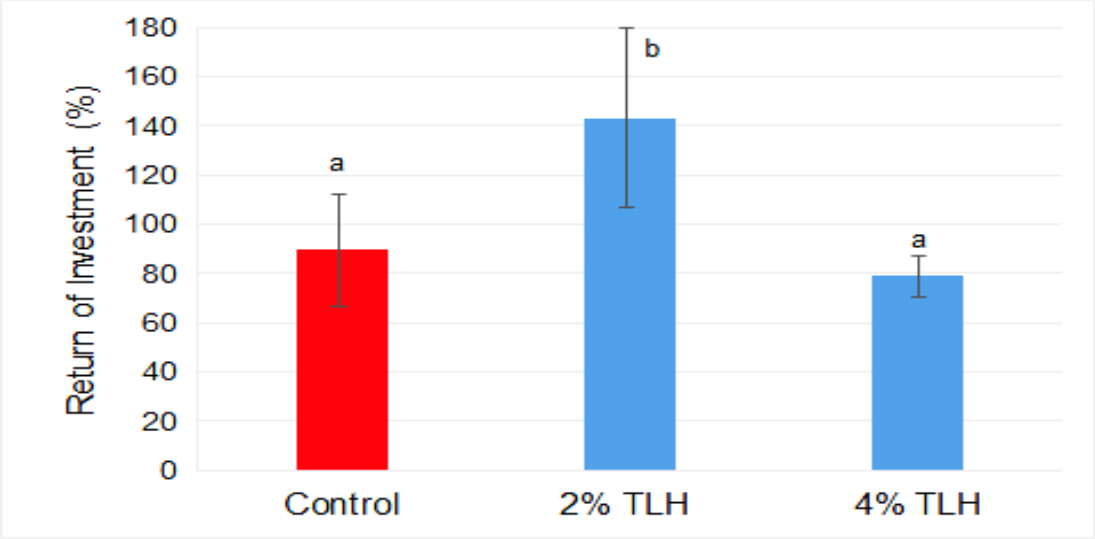


Figure 5.13: Possible return of investment of two different Tuna Liquid Hydrolysate (TLH) diet inclusion and control group, for *Litopenaeus vannamei*. Data were calculated with the average per group ± SD. Different letters mean significant differences (ANOVA + Tukey test, p=0.00851).

5.4.2 Feed peptide profile

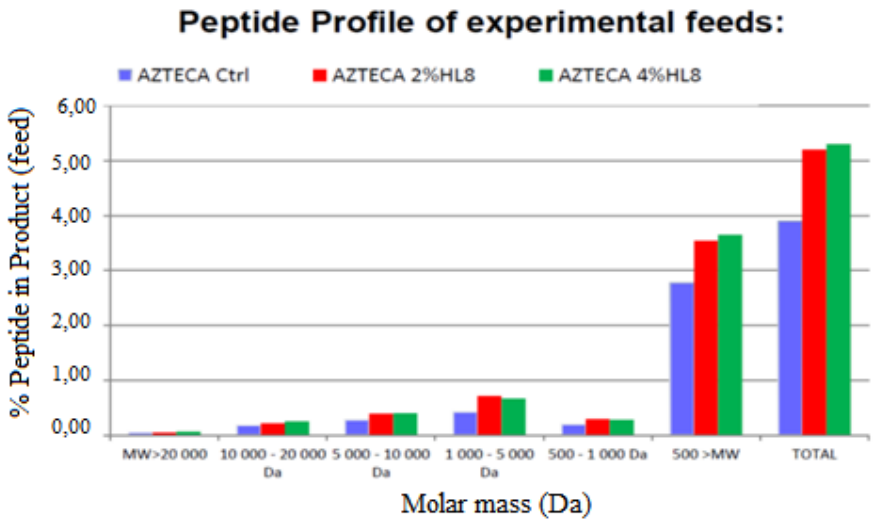


Figure 5.14. Peptide (%) in product from experimental feed and molecular weight and differences between experimental diets by molecular weight.

5.4.3 High Throughput Sequencing Results

In the present study, we used the Ion Torrent™ 16S rRNA gene-based technology to characterize the intestinal bacterial community of *L. vannammei*, in three different treatments, i.e., 0%, 2%, and 4% TLH dietary inclusions, from an amplified fragment of 350 bp. Sequencing resulted in 5’277,002 total reads. After trimming and filtration of the raw data, with the use of FASTX-Toolkit (Hannon Lab), an average of 104,582 reads were considered with high quality, ranging from 81,928 to 133,308. The Archaea were filtered to avoid interference with statistical interpretation and quantitative results. Quality-filtered reads were clustered into OTUS at 3% distance, along with each OTUs represented a unique phylotype. For all samples, Good´s coverage index was above 0.995, indicating adequate sequencing. The rarefaction curves revealed that samples from both treatments and control groups reached their saturation phase, admitting data diversity analysis (Figure 5.16).

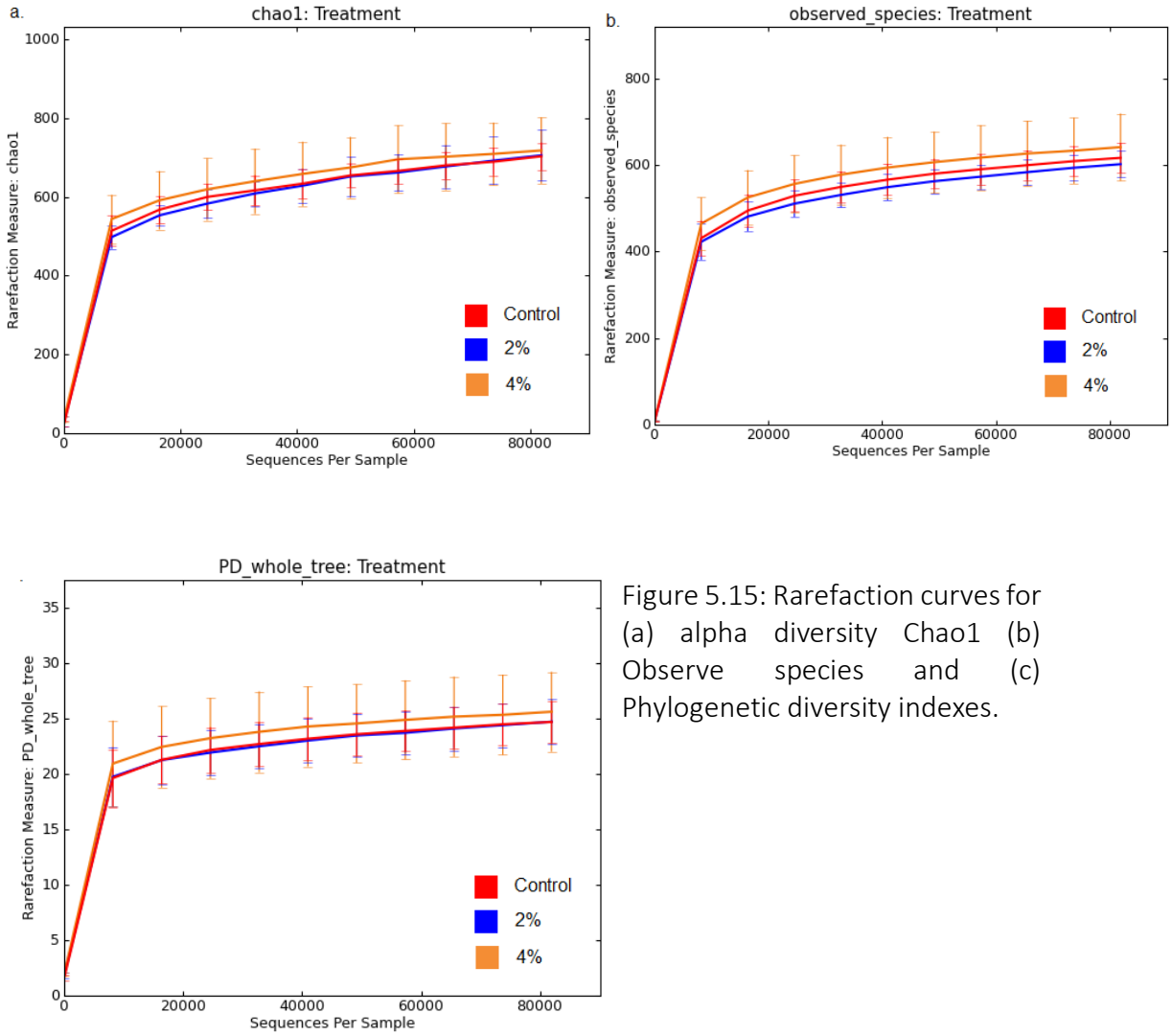


Figure 5.15: Rarefaction curves for (a) alpha diversity Chao1 (b) Observe species and (c) Phylogenetic diversity indexes.

Table 5.6 summarizes the High Throughput Sequencing results, with reads from bacteria phyla and genera between treatments, classified according to their numbers of reads and abundance. Part of this relative abundance includes the core microbiota and the microbiota associated with changes and other roles in the gut. The alpha diversity metric was calculated from the rarefaction curves from each group with the most abundant sequences obtained from OTUs at the genus level.

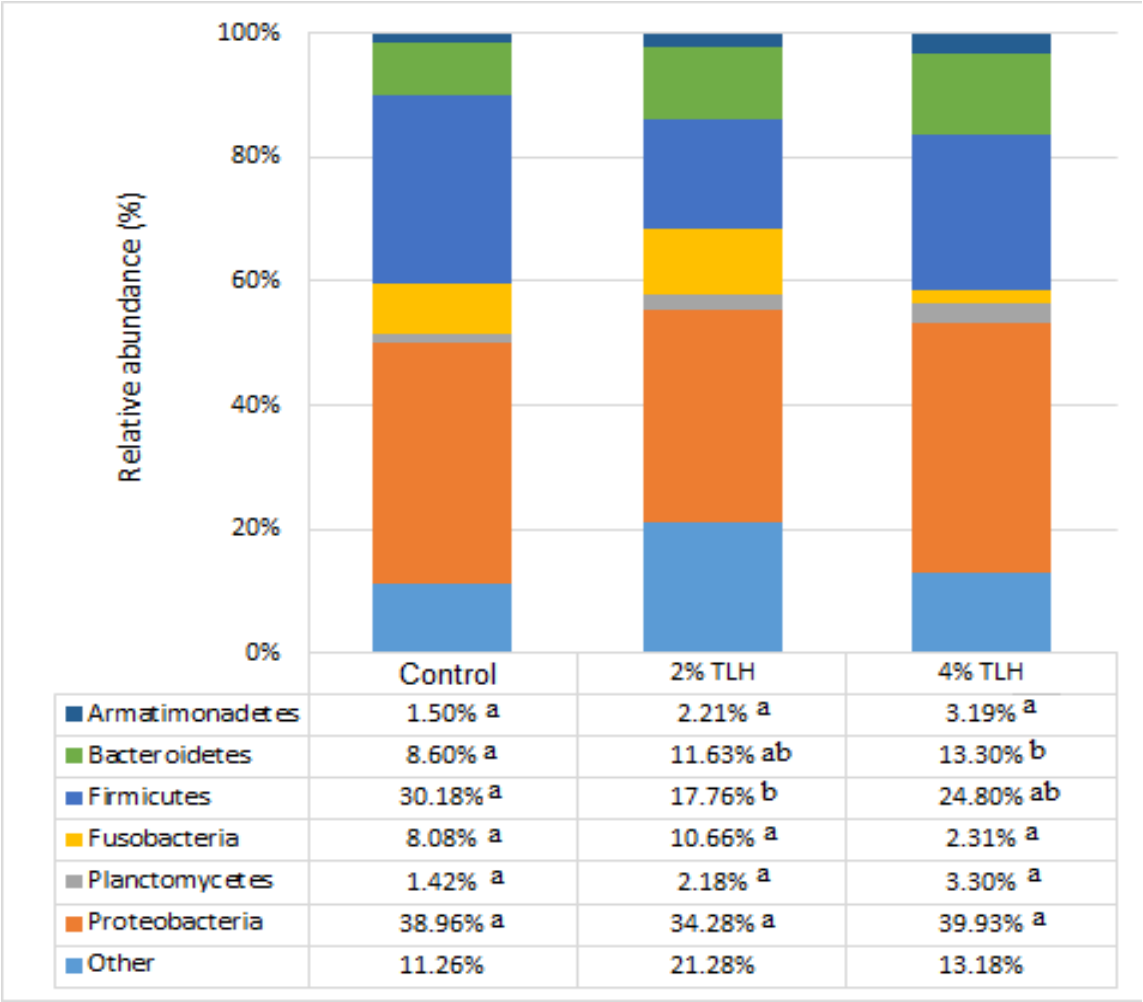
Table 5.6: Summary of High Throughput Sequencing result, showing the alpha diversity indexes of *Litopenaeus vannamei* intestinal microbiota.

	Control	2%	4%
Reads after trimming	636,699 ± 8,823	602,527 ± 9,370	643,266 ± 9,268
OTUs (%3) – Phylum level	24	29	27
OTUs (3%) – Genus level	300	312	305
Alpha diversity indexes			
Chao 1	703.06 ± 34.67	705.42 ± 64.75	717.85 ± 83.97
Observed Species	616.20 ± 34.13	601.53 ± 30.36	640.93 ± 77.59
Phylogenetic diversity	24.66 ± 1.84	24.68 ± 2.01	25.58 3.59

5.4.4 Relative abundance and diversity of gut microbiome

In terms of taxonomy and relative abundance, it was possible to distinguish the six most abundant phyla in *L. vannamei* gut microbiota (Fig. 5.17), and statistical results from ANOVA showed differences between microbiota from gut among treatments. The greatest relative abundant phylum observed was Proteobacteria, which presented similar abundance regardless of the experimental condition ($p>0.05$). Then, phylum Firmicutes was the second most abundant, with the highest percentages observed on the 0% TLH group and statistically higher than 2% TLH group ($p=0.0375$), followed by Bacteroidetes, which was statistical lower on 0% TLH treatment ($p=0.0463$) in comparison to 4% TLH group. The 4% TLH inclusion treatment showed the lower relative abundance of phylum Fusobacteria, although not significantly ($p>0.05$). In

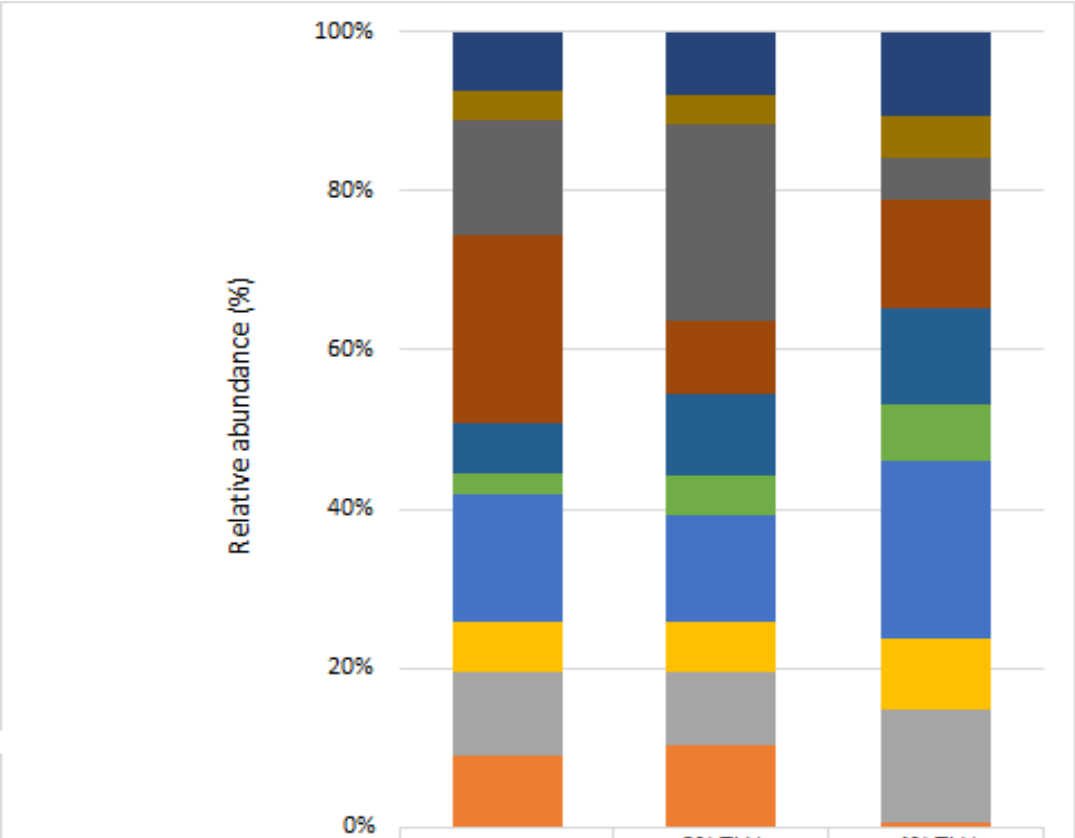
total, over seventeen phyla were detected including the dominion Bacteria and Archaea.



	Control	2% TLH	4% TLH
Armatimonadetes	1.50% a	2.21% a	3.19% a
Bacteroidetes	8.60% a	11.63% ab	13.30% b
Firmicutes	30.18% a	17.76% b	24.80% ab
Fusobacteria	8.08% a	10.66% a	2.31% a
Planctomycetes	1.42% a	2.18% a	3.30% a
Proteobacteria	38.96% a	34.28% a	39.93% a
Other	11.26%	21.28%	13.18%

Figure 5.16: Relative abundance of gut microbiota composition of *Litopenaeus vannamei* receiving two different Tuna Liquid Hydrolysate (TLH) diet inclusion group, describing the distribution (%) of bacteria, at the phylum level.

Comparatively, at genera level, it was possible to identify a total of ten bacteria with the mayor relative abundance, including *Cetobacterium, Enterococcus, Mycoplana, Lactococcus*, and *Shewanella* (Fig. 5.18). Some genera were noticed in all treatments but had an increase or decrease regarding the dietary TLH inclusion, in absolute numbers although there is no statistical difference. Genera *Lactococcus, Cetobacterium, Rhizobiales, Enterococcus,* and *Exigobacterium* were also the most dynamic in between treatments with the inclusion of TLH. We highlight that *Cetobacterium*, a relevant genus related to vitamin B12 in fish (Rodiles et al., 2018), was one of the most abundant genera in *L. vannamei* gut microbiota, higher on control and 2% TLH inclusion group, although drastically dropped down at the 4% TLH inclusion. Moreover, the genus *Enterococcus* presented a decrease in abundance according to the inclusion level of TLH in shrimp diets, from 23.79% in control diet to 9.21% and 13.74% in 2% and 4% inclusion level, respectively. On the other hand, 4% TLH inclusion treatment showed an overall higher abundance of genera *Mycoplana* and *Lactococcus*, while this same treatment presented the lowest abundance of *Shewanella*.



	Control	2% TLH	4% TLH
Agrobacterium	7.56%[a]	8.00%[a]	10.53%[a]
Bradyhizobiaceae (Family)	3.44%[a]	3.55%[a]	5.45%[a]
Cetobacterium	14.52%[a]	24.70%[a]	5.09%[a]
Enterococcus	23.79%[a]	9.21%[a]	13.74%[a]
Exiguobacterium	6.27%[a]	10.25%[a]	12.10%[a]
Fimbriimonadaceae (Family)	2.67%[a]	5.05%[a]	6.96%[a]
Lactococcus	15.86%[a]	13.40%[a]	22.36%[a]
Mycoplana	6.47%[a]	6.41%[a]	9.01%[a]
Rhizobiales (Order)	10.36%[a]	9.18%[a]	14.26%[a]
Shewanella	9.07%[a]	10.24%[a]	0.45%[a]
Other	0.01%	0.01%	0.05%

Figure 5.17: Relative abundance of gut microbiota composition of *Litopenaeus vannamei* receiving two different Tuna Liquid Hydrolysate (TLH) diet inclusion group, describing the distribution (%) of bacteria, at the genus level.

5.4.5. Similarities and dissimilarities.

Based on Weighted and Unweighted UniFrac distance, we investigated similarities and dissimilarities, by Principal Coordinate Analysis (PCoA) (Fig. 5.19). The spatial distribution of our samples revealed mixed communities, with no evident separation between treatments. Moreover, especially on Weighted analysis, results suggested that the majority of samples tend to cluster all together. Whereas the Unweighted analysis showed a more dispersed bacterial community.

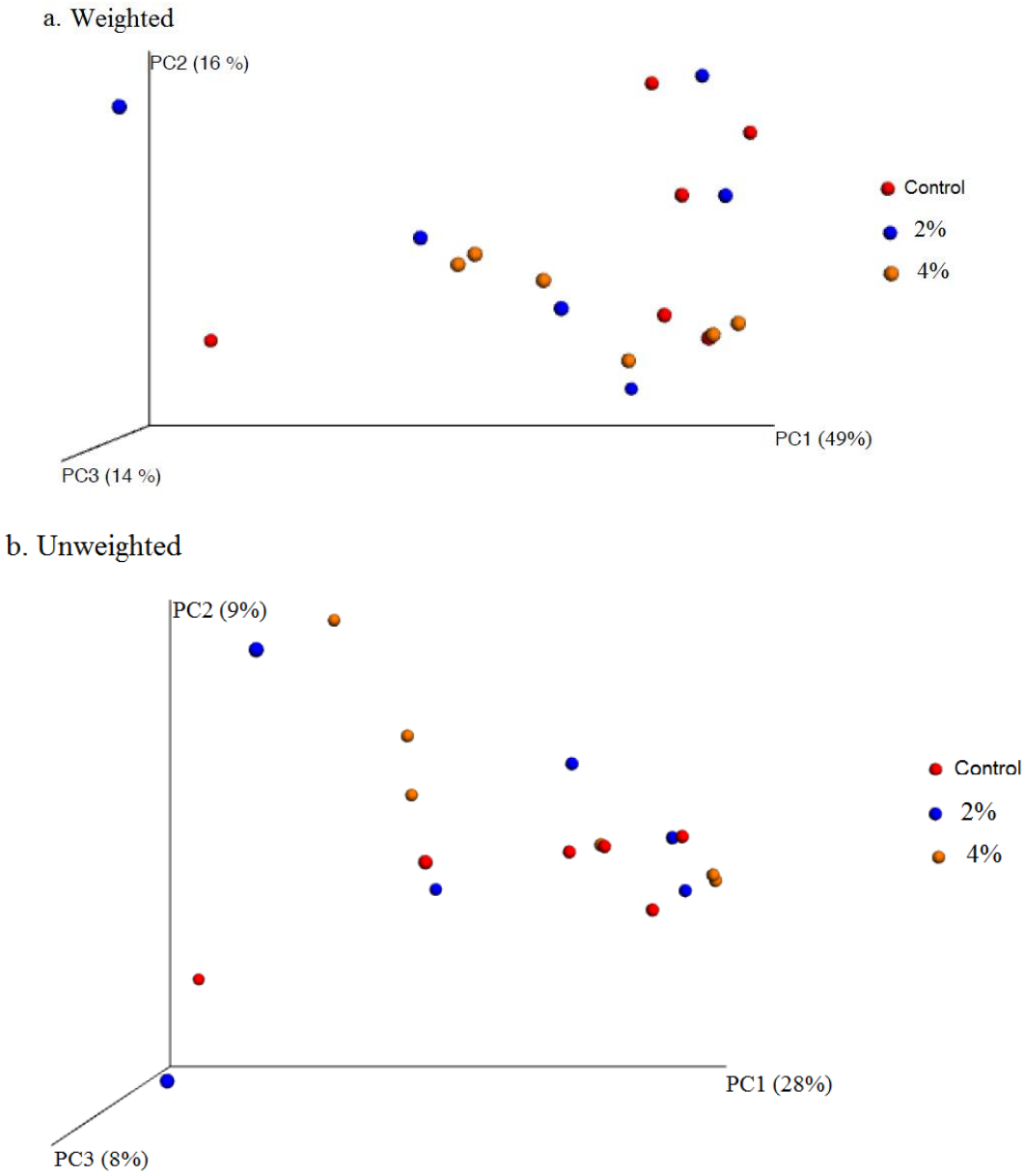
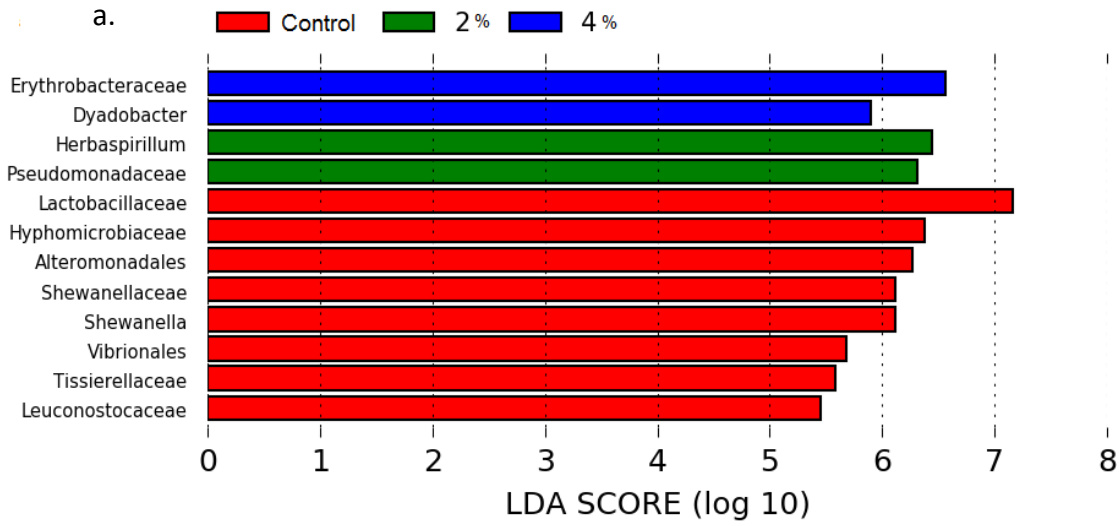


Figure 5.18: Principal Coordinate Analysis of gut microbiota composition of *Litopenaeus vannamei* receiving two different Tuna Liquid Hydrolysate (TLH) diet inclusion and control group, based on (a) Weighted and (b) Unweighted UniFrac distances.

The Linear discriminant analysis effect size (LEfSe) is a method to discover biomarkers, identifying possible taxa that have statistical significance and biological relevance in a given population, characterizing these and showing the most likely bacteria group that can explain the differences between treatments. In the current study, LEfSe revealed which taxa had differences in the relative abundance in the three groups analysed, namely 0%, 2%, and 4% TLH dietary inclusion, at the genus level. Similarly, the logarithmic LDA score measures the number of differences in the relative abundance between taxa, i.e., the effect size of each feature, sorting the differences between classes of the analysed data (Segata et al., 2011).

LEfSe method revealed distinct taxa in the gut microbiota of the three treatments of this study, all with a positive LDA score (Fig. 5.20). The gut microbiota of 2% and 4% TLH inclusion groups displayed only two differential taxa in each group. Among the 2% TLH, *Herbaspirillum* (Genus) and Pseudomonadaceae (Family) were enriched, while taxa within Erythrobacteraceae (Family) and *Dyadobacter* (Genus) were enriched in 4% TLH. Nonetheless, gut microbiota of control group presented eight distinct and enriched taxa, highlighting Lactobacillaceae (Family) with the greatest effect size, with an LDA score higher than 7.0. We also stand out other key taxa in the control group, such as Alteromonadales (Order), *Shewanella* (Genus), and Vibrionales (Order).



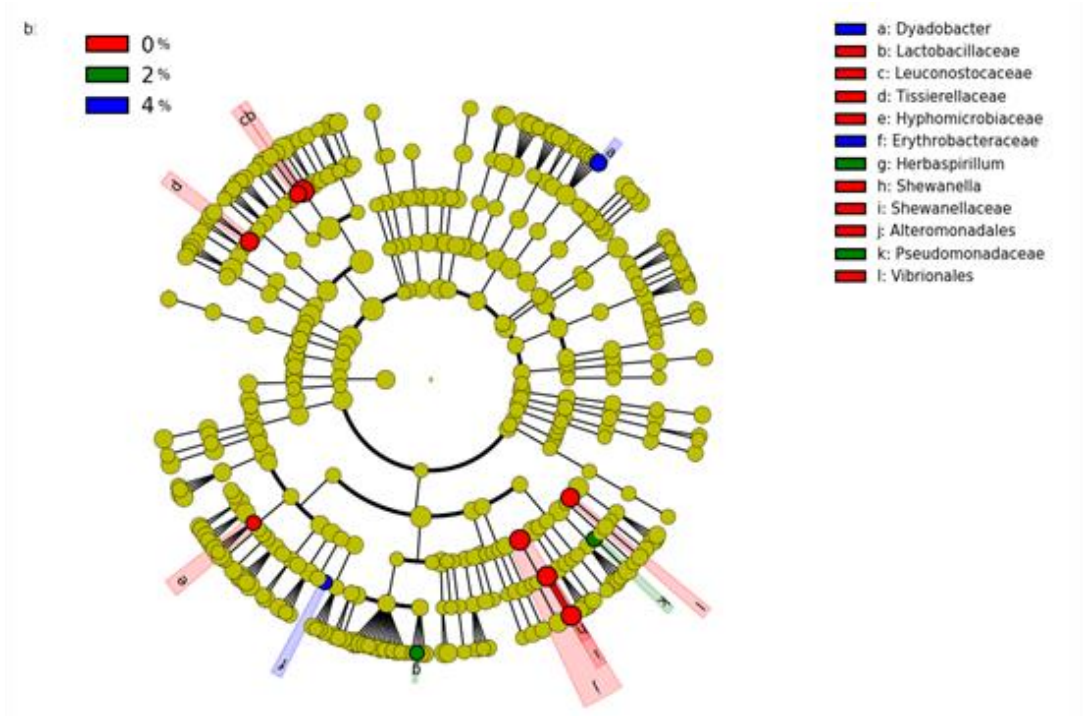


Figure 5.19: Distinct enriched taxa in the gut microbiota of *Litopenaeus vannamei*, with two different Tuna Liquid Hydrolysate (TLH) diet inclusion, i.e., 2% and 4%., and control group, at the genus level. (a) LDA score showing the effect size of each taxon. (b) Relative abundance of the five most abundant bacteria, at genus level, in order to support Lefse results. OTUs at genus level P<0.05; LDA threshold of 2.

To describe the microbiota intersection between treatments, as well as to define the core gut microbiota, i.e., to identify unique and shared OTUs presented in the intestine of *L. vannamei* and their crossing between treatments, a Venn diagram was constructed, at the genus level (Fig. 5.21). The core microbiota was comprised by 74 shared OTUs, that is, 48.4% of OTUs did not display any influence from any dietary treatments, as they were all present in shrimp gut, regardless the inclusion of TLH. All the ten most relative abundant OTUs identified at genus level were present in the core microbiota. On the other hand, 30% of OTUs were exclusively influenced by the inclusion of TLH on shrimp diet, i.e., 46 bacteria OTUs at genus level were present only in samples treated with TLH, suggesting the presence of certain bacteria genera due to the inclusion of TLH. In contrast, the control treatment consisted of 13 exclusive OTUs, indicating that 8.5% of the gut microbiota were suppressed and disappeared in shrimp with 2% or 4% of TLH diet; of these inhibited bacteria it is possible to draw attention to the Lactobacillaceae, this Family of lactic acid bacteria are a potential probitic for crustaceans (Castex; Daniels; Chim, 2014), as are the *Corynebacterium* and Flavobacteriales (Order), commonly found in gut microbiota of shrimp from commercial farms (Chaiyapechara et al. 2012).

Mexican law corresponding to NOM- 027-SSA1-1993, estipulates the managment, ethics and accepted process, transportation, storage, temperature, ice and post conditioning from fish discards including tuna trimmings. Also, temperature below -18C must be kept to avoid enzymatic activity, bacteria presence and decomposition, trace metals such as Cadmium (Cd) < 0,5, Mercury (Hg) 1,0, Lead (Pb) 10. 9 (NOM- Mexico-Fisheries,1993)

The 2% TLH treatment consisted of ten OTUs (6.5%), at the genus level, which were only found in this treatment, including the relevant probiotic bacteria *Bacillus*, and shared six OTUs (3.9%) with control group and 13 (8.5%) with the 4% TLH. In contrast, the 4% TLH presented 23 (15%) unique bacteria genera, including *Pseudomonas* and *Nitrobacter*, also sharing 14 OTUs (9,2%) with the group control. Moreover, 4% TLH treatment presented the greatest number of identified OTUs, being the most abundant in terms of bacteria presence and showing 17.7% of similarity with the other two treatments, not counting the core microbiota.

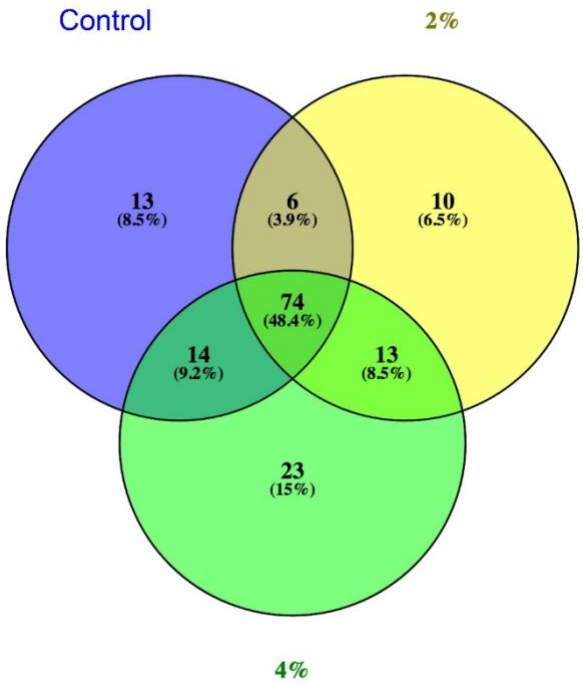


Figure 5.20: Venn diagram showing unique and shared OTUs (Operational Taxonomic Units) in gut microbiome of *Litopenaeus vannamei* that received different Tuna Liquid Hydrolysate (TLH) diet inclusion, i.e., 2%, and 4%, and control, at the genus level.

5.5 Discussion

In view of the growing demand for alternative protein sources for aquaculture feeds, products such as Tuna Liquid Hydrolysate (TLH) appear as a possible choice to be used as a fish by-product with antioxidant activity as a supplementary feed additive. Nowadays, discards from industrial fisheries is estimated to be up to 10 million tons/year (Zeller et al., 2017), requiring with urgency a better utilization of all these discarded by-products that have great commercial potential this has important environmental significance for the global food generation.

Fundamentally, the primary components to be considered when analysing a possible new ingredient to aquaculture are digestibility, palatability, and nutrient utilisation as see as growth performance (Glencross; Smith, 2014). The present research tested a commercial TLH dietary inclusion and, as our study did not aim at the full ingredient evaluation process as a major dietary protein source to replace fishmeal, the assessment of protein digestibility was previously examined by Nutrimentos Acuicolas Azteca SA de CV company, resulted in satisfactory range ~ 86 %, under a systematic analysis of peptine at 0.002.

In respect to palatability, it is paramount to emphasize that the feed intake in shrimp is a challenging parameter to measure, being in this research reported as the apparent Feed Conversion Rate (FCR), i.e., the efficiency in converting feed intake into unit gain or output. Diets with 0% (control) and 2% TLH inclusion resulted in the best FCR

observed, with the 2% TLH inclusion group showing 4% TLH lower FCR that needs to be confirmed in subsequent studies.

The Feed Conversion Ratio (FCR) is an important parameter in a commercial shrimp farming system, in which animal nutrition costs represent a high portion of the total investment. Particularly in intensive systems, shrimp nutrient intake is totally dependent on aquafeed, as there is almost no natural feeding. An optimum FCR results from the combination of several factors, such as diet formulation, facilities, animal genetics, health status, and management. In this work, the only variation observed was the diet formulation, where 0% and 2% TLH inclusions presented commercially attractive FCR. Hernández et al. (2004) reported similar results, when juvenile *L. vannamei* were fed with fish meal with co-extruded wet tuna and wheat and presented excellent digestibility and palatability. On the other hand, the inclusion of 4% TLH did not yield a positive improvement in the growth and feed utilization metrics.

Moreover, with a high-quality aquafeed, it is possible to achieve not only the greatest FCR, but also adequate growth and weight gain, high survival, and satisfactory productivity. Further, the different TLH inclusion levels permitted the analysis of potential interactions between the diet ingredients (Glencross et al., 2007). Thus, it was possible to measure the nutrient utilization through the mensuration of growth rate and weight gain, i.e., the capability of the shrimp to well utilize the feed ingredient, converting efficiently into new biomass. Regarding growth and weekly weight gain, despite no statistical evidence between treatments, it is worthy to address that different TLH inclusions did not lead to any disadvantage to these zootechnical

parameters. In other words, the inclusion of TLH in shrimp aquafeed can be considerated appropriate due to the proper use of the dietary nutrients for animal growth. Nonetheless, we recommend follow-on research with additional methods (Hemocyte analysis, inmunne response, ammino acid profile from experimental feeds) in order to corroborate the satisfactory nutrient utilization when adding TLH in shrimp feed, such as an evaluation of immune responses and associated effect on embolism and physiological responses. (Glencross et al., 2014; Sánchez-Muros et al., 2018; Rahimnejad et al., 2019; Xie et al., 2019)

In regard to shrimp survival, significant differences were found between treatments. Diets with 0% and 2% TLH inclusion showed the best survival values, above 92%, while 4% TLH diet presented the lowest survival, with a value of 84%. Noteworthy, all treatments maintained excellent survival values, always above the commercial standards for an intense and advanced technical shrimp farming approach.

Nguyen, Pérez-Gálvez & Bergé (2012) described similar results, when testing soluble and insoluble protein powder from hydrolysis of tuna head in *L. vannamei*, reaching 82% to 97% of shrimp survival. These latter authors basal other conclusions not only on survival but also on gain weight, growth, FCR, and protein efficiency ratio, authors concluded that the supplementation of hydrolysates from tuna head showed a positive effect on major the zootechnical parameter of *L. vannamei.* Likewise, Hernández et al. (2004) stated it is possible to include a mixture of co-extruded wet tuna plus corn meal by up to 40% in shrimp diet, with similar performance in comparison to other aquafeeds, while Valle et al. (2015) specified that the ideal substitution level of fish

protein hydrolysate (residue from tilapia carcass) for *L. vannamei* post larvae diets are between 15.16% and 16.5%, also resulting with ideal zootechnical parameters. Similarly, the present study demonstrates that the inclusion of TLH in shrimp feed contributes to excellent zootechnical performance, with the inclusion of 2% the most appealing for freshwater juvenile *L. vannamei* and providing optimal performance under our conditions.

The main goal of all commercial shrimp farming is to attain a productive and profitable production scenario. Animal nutrition is one of the most relevant inputs to increase and to achieve a sustainable production level. Thus, when a new feed ingredient is proposed, it is indispensable to evaluate the economic cost of the proposed product. Our results confirmed that the use of TLH in shrimp feed not only can increase up to 10% the productivity but also it may bring an increase on the return of the investment, especially a 2% TLH inclusion due to better performance quality pellet in general. Therefore, the present study, based on zootechnical performance and economic inferences, endorses the inclusion of 2% Tuna Liquid Hydrolysate on shrimp feed, in an intense freshwater shrimp farming.

Mantaining the intestinal microbiota homeostasis is paramount to promote an adequate health status of shrimp. However, despite *L. vannamei* being the most cultivated shrimp worldwide, there are few studies regarding a possible gut modulation due to different diet formulation or new ingredients. Withal, the study of gut modulation is essential against new ingredients that include it's imperative to characterize the potential characteristics of potential from novel feed ingredients in

the regime. The confirmation that they do not impose an unwanted microbial dysbiosis is necessary, many ingredients contain antinutritional factor and ingestible carbohydrates that could lead to microbial imbalance.

Concerning of richness and diversity indicies resulted from DNA sequencing from gut microbiota, in general the High Throughput Sequencing (HTS) presented a large number of reads and OTUS. The high values of Good´s Coverage index, above 0.995, confirmed that the bacterial DNA sequencing was reliable in the reads alignment, seeing that the higher Good´s Coverage, the more trustworthy the quality position of extracel DNA. Additionally, the rarefaction curves reached their corresponding plateau, which indicates that both the richness and the bacterial diversity of the analyzed samples were determined, therefore we can be confident on our data.

Furthermore, superior values of Chao indexes were obtained in the shrimp samples from the trial. The species richness is the total number of species in a sample (Gotelli; Chao, 2013). The Chao method estimates the total richness, considering the species occurring in one and only one sample, i.e., the number of species represented by only one individual in the samples (singletons). Chao also considers the species occurring in two and only two samples, i.e., the number of species with only two individuals in the samples (doubletons). In other words, this richness index estimates the total number of species in a given community, and if a sample contains many singletons, the Chao1 index will estimate greater species richness due to the higher number of rare OTUs (Colwell; Coddington, 1994). On its turn, the count of observed species is a species richness estimator that takes into consideration the count of unique OTUs in each

sample. In addition, the phylogenetic richness estimator considers the phylogeny of the microorganisms to estimate diversity, i.e., the estimator observers the degree of correspondence among a group of species in a sample (Baltanás, 1992; Gotelli; Chao, 2013).

In relation to taxonomy and relative abundance analysis, six phyla were distinguished as the most abundant in *L. vannamei* intestine. The phylum Proteobacteria was the predominant among all samples, regardless of the level of TLH inclusion on the feed. The second phylum most abundant was Firmicutes, regardless of the treatment. These observations are consistent with previous research work on different crustaceans (Cheung 2015), such as *L. vannameii* (Huang et al. 2016), the black tiger shrimp *Penaeus monodon* (Rungrassamee et al. 2014), the Chinesses shrimp *Penaeus chinensis* (Liu et al. 2011), the oriental river prawn (*Macrobrachium nipponense*) (Tzeng et al. 2015), and the Chinese mitten crab (*Eriocheir sinensis*) (Ding et al. 2017). We also highlight the abundance decrease in the phylum Fusobacteria in the 4% TLH inclusion treatments. This significance of this finding warrants consideration in relation to shrimp health status mentioned in this document as Shrimp fitness.

However, it is also relevant to have in mind that the salinity can strongly influence the gut microbiota of *L. vannamei* (Zhang et al. 2016); thus, all results presented in this study should be related to a brackish water system scenario. Moreover, typically challenges in low salinity on shrimp farming with 80 PL/m^3 stocking density can be related to bacterial infections and other pathogenic challenges. Based on our farm

experience and observation, some taxa from the family *Vibrionacea* and other pathogens have been reported in the last five years at certain shrimp size, commonly between day 30 to 60, when total biomass is close to 600 g/m² and the feeding ratio is close to 20 grams/day/m^2 of shrimp feed.

When analyzing the relative abundance at the genus level, we observed that the most abundant genus differed between treatments. In the control group, genus *Enterococcus* was the most prevalent, followed by *Lactoccocus* and *Cetobacterium*. Differently, animals that received a 2% TLH dietary inclusion presented *Cetobacterium* as the most predominant genus, followed by *Lactococcus* and *Exiguobacterium*. Finally, *Lactococcus* and Rhizobiales (Order) was the most abundant at the 4% treatment. In fact, at phyla level, phyla *Firmicutes* were more prevalent at 0% and 4% TLH treatments, while *Fusobacteria* were greater at the 2% group. Thus, it is possible to disclose that different TLH dietary inclusions modulated the posterior gut microbiota of *L. vannamei* in terms of relative abundance, both at phyla and genera level.

Some aspects of the relative abundance deserve a better discussion. For instance, some results apparently had no interrelation between treatments. *Cetobacterium*, for example, although significantly high in the 0% (14.52%) and 2% (24.70%) TLH groups, it was drastically down at the 4% inclusion level (5.09%). *Cetobacterium* is intrinsically associated with the production of vitamin B12 (cobalamin) in fish (Tsuchiya; Sakata; Sugita, 2008). Moreover, when the species *Cetobacterium somare* is present in the gut microbiota of fish, there is no need to supplement aquafeed with vitamin B12

(Merrifield; Ringo, 2014). In shrimp, some authors have associated the abundance of *Cetobacterium* genus with the high stocking density (Zheng et al. 2017). However, little is known about a possible role of this genus in the production of cobalamin in crustacean, being that a promising research subject.

The *Exiguobacterium* genus had a significant increase with the inclusion of TLH in accordance with the addition level: control (6.27%), 2% TLH inclusion (10.25%), and 4% TLH inclusion (12.10%). According to Orozco-Medina; López –Cortés; Maeda-Martínzez (2009), the specie *Exigoubacterium mexiacanum* showed positive results on artemia larvae development, i.e., a beneficial bacterium with a positive effect on crustacean development. Sombatjinda et al. (2014) reported that *Exiguobacterium* may improve survival and growth in *L. vannamei.* Additionally, *E. arabatum* isolated from gut of healthy *L. vannamei* revealed to have potential probiotic functions (Cong et al. 2017).

Specifically, concerning the 4% TLH treatment, it presented significant difference in some genera, such as an increase in *Lactococcus* (22.36%), Rhizobiales (Order) (14.26%), *Exigobacterium* (12.10%), and *Agrobacterium* (10.53%), which were significant higher in increase and abundance. On the other hand, this treatment showed a significant decrease on *Cetobacterium* (5.09%) and *Shewanella* (0.45%). In comparison with the other microbiota profile, the 4% TLH group had a significance ratio of *Lactococcus,* suggesting that the inclusion of 4% TLH may lead to a greater increase of this probiotic genus in the gut. However, this treatment also resulted in a much lower abundance of *Shewanella*. *Lactococcus* and *Shewanella* are one of the most relevant

phylogenetic lineages of probiotic used in crustacean aquafeed (Castex; Daniels; Chim, 2014).

Nonetheless, the 2% TLH treatment resulted in high abundance of *Lactococcus, Shewanella*, and *Exiguobacterium*, in addition to presented the highest relative abudance of *Cetobacterium*. Therefore, the 2% TLH dietary inclusion revealed to be the best TLH inclusion to *L. vannamei* aquafeed, for stablishing a healthier gut microbiota. Apart from this evidence, further studies with different inclusion levels of TLH may bring extra data to support this data with current fidings.

Additionally, a Venn diagram was constructed in order to compare the number of bacterial OTUs, at the genus level, between the different TLH dietary inclusion treatments. The diagram revealed a core microbiota composed of 48.5% OTUs, i.e., the bacterial community not influenced by any inclusion of TLH, independently of the absence or presence of this ingredient in the analyzed aquafeed. All the ten most relative abundant OTUs, at the genus level and described before, were found composing the core microbiota, including the species *Cetobacterium somerae*, associated with the production of vitamin B12, also discussed earlier. Thus, the core microbiota is composed, but not exclusively, by *Enterococcus*, *Lactococcus*, *Cetobacterium*, Rhizobiales, and *Shewanella*.

It was exclusively found in the control group, that the lactic acid bacteria Family Lactobacillaceae, together with other bacteria, such as *Corynebacterium* and Flavobacteriales were suppressed in the other two treatments that received TLH in

their diet. Although it was not feasible to affirm categorically the reason of this inhibition, we can infer that it may be related to an effect of the type of diet in the gut microbiota (Ingerslev et al. 2014; Huang et al., 2018), i.e., the TLH dietary inclusion may select the bacterial community. Nevertheless, the PCoA analysis revealed that the shrimp gut bacterial community did not formed undoubtedly different clusters, i.e., there was not a clear dissimilarity between treatments. Thus, further studies are necessary to confirm our argument as confirm this hypothesis.

Additionally, 30% of the OTUs were exclusively found composing the gut microbiota of animals fed with TLH. The 2% TLH inclusion group was the only treatment presenting *Bacillus*, a significant bacteria genus with probiotic effects (Castex; Daniels; Chim, 2014) and with several digestive enzymes that can improve animal digestion and feed absorption (Buruiana et al. 2014). Further, a dietary supplementation with *Bacillus licheniformis* can result in increased growth and better immune responses in *Macrobrachium rosebergii* (Kumar et al. 2013). Similarly, bacteria *Pseudomonas* and *Nitrobacter* were only observed in the 4% TLH inclusion group. Although some species from genus *Pseudomonas* can be opportunistic pathogens, such as *P. aeruginosa* (Hindu et al. 2018), some other species belonging to this genus show probiotic effects (Castex; Daniels; Chim, 2014), such as P. M174 and P. M162, with antimicrobial agents (Korkea-aho et al. 2011; 2012). Finally, *Nitrobacter* is a nitrifying bacteria, also classified as a bioremediator that regulates the microbiota of the pond water, controlling pathogenic bacteria and mineralizing the organic waste in the aquaculture water tank (Cébron; Garnier, 2005; Kumar et al. 2016). This might have importance for close (RAS) type sistems depending on the effective biofiltration.

It appears that the TLH dietary inclusion in *L. vannamei* aquafeed established selective pressure in the intestinal community, affecting the microbiota composition also in terms of distinct, unique and shared OTUs. Moreover, LEfSe analysis showed that the inclusion of TLH in shrimp aquafeed had a significant effect on the composition of distinct taxa. In other words, the gut microbiota changed and trended to present distinct taxa in regard to the diet inclusion of TLH.

Certainly, the use of aquaculture by-products is crucial to promote increasingly sustainable aquaculture, both in terms of eco-friendly production and commercial viability. The use of Tuna Liquid Hydrolysate a functional aquafeed ingredient suppling both demands since it has been neglected until now, without an adequate utilization, despite de fact that it is high-quality protein source. However, the addition of new ingredients in animal diet brings challenges such as increasing the complexity of animal nutrition. *De facto*, the addition of TLH in shrimp feed influences zootechnical parameters and modulates the composition of the intestinal microbiota. Favourably, the present study concludes that the dietary inclusion of TLH results in adequate growth, weight gain, and survival, besides beneficially modulate freshwater *L. vannamei* intestinal microbiota. Moreover, the use of aquafeed enriched with TLH results in higher productivity and greater return of investment at a rate of 1 to 3, i.e., it is economically attractive, besides to maximize tuna by-product market value. This is an important fact towards significantly reducing our dependence on antibiotics and the growing threat of antimicrobial resistance that is a major global issue. The transparency

of the food chain and efficacy relating to prophylactic approaches is a main driver for the use of natural dietary additives conferring functionality.

5.6 Conclusions

1. The dietary inclusions of TLH at 2% inclusion level results in optimum growth, weight gain, survival, and feed conversion ratio of *Litopenaeus vannamei*. Moreover, it also contributes to higher productivity and greater return of investment.

2. TLH dietary inclusion affects zootechnical parameters partially by modulating the intestinal microbiota; and increases the presence of beneficial bacteria in the gut.

3. The results provide evidence of intestinal bacteria community modulation with the use of novel feed additives like TLH, which increases the relative abundance of shrimp gut microbiota and significant changes at Phylum and Genus levels.

4. The inclusion of TLH at 2% and 4% within the diets shows significant differences in the composition of gut microbiota of *L. vannamei*, with beneficial bacteria strains becoming autochthonous. These beneficial bacteria can reduce the effect of pathogenic bacteria coming from the shrimp ponds during the production cycle by out competing.

5. The TLH promoted as a sustainable ingredient and can improve shrimp health and gut microbiota during productive shrimp cycle under commercial conditions

6. TLH dietary inclusion for *Litopenaeus vannamei* modulates the gut microbiota, increasing the productivity.

7. The use of TLH cooperates with a better utilization of a sub product of the aquaculture industry, adding marketing value to tuna residue.

8. The functionality of some bacteria genera that can be linked to probiotics effects and can help with the performance and survivability of the shrimps.

9. Been the 2% inclusion level the best in terms of relative abundance and with the presence of the beneficial bacteria its suggested for further studies to compare the inclusion of 1% and 1.5 % against the 2% to underline the precise inclusion level and the cost benefit for the final consumer.

CHAPTER 6. General discussion

6.1. Overview of the programme of research

This programme of research was primarily focused on enhancing the health and quality of intensively farmed shrimp in Mexico and of significance in wider Latin America. Through the implementation of *in vivo* commercial trials with floating cages in shrimp ponds, dietary supplementation with selected additives within typical formulated feeds as used in the region was analysed. These comprised commercial β-glucans, yeast/herb mixtures and a tuna hydrolyzate product at defined inclusion rates in a series of experimental diets. Parameters relating to shrimp production, performance and health were measured with a view to utilisation of the feed supplements in commercial practice.

In total, three separate trials were performed during the four years of shrimp production cycles and experimental work based at a site in Colima, Mexico. In this facility, bouoyant floating cages were used to simulate commercial conditions in shrimp ponds and to provide replicate containment systems for shrimp studies at a controlled level. Using a standard experimental protocol, shrimp were stocked and screened to similar mean initial starting weight of 3.00 ± 0.25 g and randomly distributed into each unit. These were fed with 9” circular feeding trays 2 times a day for optimum feed consumption and monitoring of behaviour and activity.

Multiple techniques were used when handling shrimp, weighing protocols, and observations of shrimp fitness. After typical 8-14-week trials shrimp where harvested for analysis and sampling. A few attempts to challenge the shrimps under extreme conditions were performed with good overall results. However, poor experience when challenging and collecting samples from hemolymph, hepatopancreas, respiratory burst messurements, indicative of oxidative stress did not yield good results (where two of the trials were discharged due to the low survival and lack of data collected).

Most of the laboratory work was performed at the University of Plymouth, UK with the support of experienced technicians under controlled areas on the campus. Different equipment and techniques were implemented when analysing biologial material in the Food Science Laboratory compliant with the parameters detremined for complete assessments in each of the trials. The generic nutrition techniques and equipment implemented were primarily Soxhlet, Kjeldhal apparatus and muffle furnaces, drying ovens and bomb calorimetry for routine Proximate Analayis (AOAC), Specialised techniques employed electrophoresis chromatography, DNA extraction purification, PCR, DDGE, also molecular based line analysis such as Next Generation Sequence and bioinformatics with Qimme as advanced procedures used in specific shrimp feeding trials as describe more fully in each relevant preceding chapters. The project also utilised general histological processes such as fixation of samples, microtome and specific staining of tissues derived from shrimp obtained at the end of feeding trials.

6.1.1. Improving shrimp health and performance

This current research presents an integrated blend of 'state of the art' techniques that have been applied in aquaculture to enhance aquatic animal health (fish and shrimp) health though prophylactic strategic use of functional feeds. With our aim towards improving shrimp production and fitness, we were mindful of the various husbandry scenarios found in shrimp culture and endeavoured to incorporate these within our assessment protocols from the shrimp farm.

These all fitted within the first experiment that was clearly designed to set the pathway for future applied research with shrimp nutrition. Conditions for rearing were adjusted to the cages and feeding strategy, management, pond depth, stocking and other implications whilst running trials for over eight-week periods to achieve optimum conditions for sufficient growth.

The second and the third experimental trials were similar, but with the purpose to show improvement in survival, growth, length, and fitness and gut modulation. We adjusted the number of weeks, density of animals, and location of the cages in the ponds accordingly. It should be noted that all feeds used in the trials were manufactured by a domestic company in Mexico. These diets gave excellent performance and served as a baseline for the addition of the respective feed additives tested in the programme of work.

More specialist approaches were used for the investigations concerning the evaluation of the Yeast and TLH used in our trials. For these experiments, we applied both nutrigenomics and the NGS, being a great opportunity to detect bacterial changes in the gastrointestinal tract and aligned with performance and correlations in health and survival. This provided a unique insight into the potential effects although much more refinement of the methods will be required in future.

The current work was envisaged as a model for an ecological or green biotechnological solution for the formulation of improved shrimp diets. Our objective for better gut health and immune response against pathogenic bacteria and other challenges in the ponds were paramount in the design of our studies.

6.1.2 Modulation of gut microbiome and health

When analysing the bacteria abundance from both analysis we found a significant increase in some of the bacteria genera, as seen *Bacteroides* had an increase in both trials regardless the feed additive, the majority of the bacteria genera was not corelated to the trials, as seen with other bacteria as *Bacteroides* a reverse trend was observe with the inclusion of these FFA, although no correlation was observe in the abundance of some bacteria from phylum Firmicutes, where the presence of the YAH display an increase and the second group with increase inclusion had a decree suggesting that the bacteria modulation in the shrimp gut is

very sensitive and can either decrease or increase according to inclusion rate and level of feed additive.

From our experience as a farmer, the same situation can be seen in the ponds, a multi variable of bacteria presence can be observe by standard culture-based methods when increasing or decreasing farm factors, such as density, temperature, water exchange, feed quality and climatic influence. Making this a multifactorial chain that is continuously changing over a period while in the shrimp is growing in the ponds.

Pond conditions during the experimental trials

Some of the major parameters affecting bacteria populations in the gut and also in the ponds are, density, salinity, feed quality, management of the ponds and disinfection on the first hand, secondly the temperature, oxygen, microalgae and natural plankton, inputs from sources such as nitrogen, phosphorus and carbon sources and natural environmental changes such as hurricanes, cold storms and global warming.

General Chapter comments

In chapter 3, four types of β-glucans where evaluated with different inclusion levels. The study demonstrated satisfactory results in terms of survival, considering the long cycle (140 days) and the high density inside the cages (>1.3 kg/m^3). Excellent shrimp fitness (carcass, colour, Hepatopancreas lipid content, gut morphology, mobility and capacity to tolerate stress) was also observed, and even the stress trials with abrupt salinity change from five to 33 ppt for three hours did not affect the shrimp, which presented, at the end of the trial great survival. However, feed conversion ratio and specific growth rate were found to be above standard for shrimp farming, thus further research needs to be performed to establish inclusion rate and prophylactics method to optimize the use of β-glucans with shrimps.

In addition, the economic analysis (including FCR, total cost of feed, cost per kilogram of shrimp, market price and profit) from this trial were significant in comparison with the performance of the shrimp but the survival was outstanding, suggesting that additional analysis needs to be performed to adjust dosage and remain competitive with industry standards and cost-effective values. Differences in shrimp's carcass, lipids, protein and ash were not found in-between treatments with the inclusion of four β-glucans, where proximate analysis at the analytical laboratory from Plymouth University resulted in no difference.

A strong recommendation we advocate is to include this feed additive in shrimp micro diets (< 200 micron in size) for early stages in order to develop and establish bacterial colonization of the gut, even before the shrimp post larvae have been dispatched and shipped from the hatchery, for three main objectives: gut colonization, gut structure improvement and to recover indigenous microbiome after been treated with antibiotics during the larval stages wich reduces the microbiome abundacy.

In chapters 4 and 5, we performed the analysis of the gut microbiota of the shrimp, through next-generation sequencing, based on the sequencing of the 16S rRNA. Next-generation sequencing techniques among traditional techniques help to evaluate shrimp gut health. They also have demonstrated that bacterial infections can be a complex and often higher diversity and richness than previously thought. Moreover, gut bacterial communities also appear to have similarities with the shrimp ponds, where feed ingredients and feed additives can modulate gut microbiota. The current work demonstrates sensitive microbiota when adding feed additives with notable increase of beneficial bacteria where survivability was above commercial standards. Future work must address the metagenomic profile of the microbiome in the gastrointestinal tract to verify actual functionality based on then full DNA profile as these cannot be addressed using the classical 16S rRNA approach used in our and most other investigations on fish and shrimp. One criticism is that our interpretation of functionality of the various bacteria identified in our studies are speculative and limited.

Shrimp microbiome

Regarding the changes in the gut microbiota in chapter 4, intestinal colonization reflected positive results when adding the YAH at 0.5% inclusion, highlighting the largest increase in beneficial bacteria genera. However, shrimp did not have significant differences in terms of performances, as SGR and FCR were similar. The core microbiota composition from this trial was analogous and confirmed with other related papers in shrimp microbiome derived from healthy shrimps. Alpha and *B* indexes, related to diversity and richness, revealed no major differences between treatments and control. The most abundant bacteria genera related to the inclusion of YAH was *Cetobacterium* follow by *Sphingobium* and *Bacillus.* Some bacteria were not affected at all by the inclusion of YAH probably because they were part of the established core microbiota, such as *Lactobacillus* and *Lactococcus*. PCoA analysis revealed that 1% treatment presented a spatial separation from control, being dissimilar to it, then the gut microbiota of this treatment was very influenced by the YAH diet inclusion, whereas 0.5% treatment revealed to be more dispersed and similar to the control groups.

Perhaps future studies might wish to investigate the possible effects of synergistic indigenous bacteria (symbiotic bacteria) related to the pond microbiome dynamics, feed sources, and natural productivity that are little understood. Although the presence of *Proteobacteria* was evident with the inclusion of YAH, no difference was found in the performance such as SGR, but total yield and shrimp survival were significantly higher ($P<0.05$) among treatments with YAH. Possible benefits from the inclusion of YAH inclusion under different circumstances such as short-term supplementation and or during periods of stress should be subject to future

studies. The results would suggest that some bacteria genera, such as *Exiguobacterium* and *Vibrio*, appear to colonize the mucosa layer for the posterior part of shrimps when adding YAH.

Regarding chapter 5, the body of our research suggested that there is a potential role for sustainable feed additives in shrimp aquaculture. We are looking towards the beginning of creating a new generation of feed for shrimp that can modulate intestinal microbiome with the use of domestic ingridients and tuna byproducts. This is the commencement of further research for bacterial role and associated gene expression studies to minimize the impact of pathogenic agents. The use of small molecular fractions (peptides and oligopeptides) that than can improve shrimp health and be an effective method to be used as preventive management in stressful and infectious conditions is a new frontier for the expansion of sustainable aquaculture globally.

However, to extend the benefits of our research, special consideration is required to optimize functional feeds additives under various conditions. Such strategies must involve testing a wider range of inclusions levels, shrimp production stages, prior to disease infection and post challenge situations. By increasing competition within the intestinal tract, it is possible to reduce the establishment and colonization of potentially pathogenic bacteria and allow competitive suppression of infective bacteria leading to disease outbreaks.

Unfortunately, the TLH inclusion study showed no significant differences were found between treatments in terms of performance, feed conversion ratio (FCR), specific growth rate (SGR),

and weight gain. The explanation for the high FCR on this trial is also similar to the chapter 1, suggesting a miscalculation in the feed that was consumed, uneaten feed monitoring, low protein, lack of bottom detritus and high temperatures under summer months that can bring false information when calculating feed consumption. Additionally, the density inside the cages was above industrial parameters and this could lead to poor feed conversion ratio and SGR.

Our study revealed that the dietary inclusion of TLH on shrimp feed may lead to superior shrimp zootechnical parameters, contributing to optimal inclusion. Moreover, the TLH diet inclusion positively modulates shrimp gut microbiota, without intestinal dysbiosis. Based on our results, the TLH is a viable and sustainable ingredient to be used on shrimp production cycles, contributing to better utilization of this tuna by-product. Thus, further analysis and challenges studies with pathogenic microorganisms may validate the use of the new generation shrimp feed in large scale aquaculture farming.

6.2 Future work

Future work must consider indigenous bacteria from gut and from the soil ponds to understand bacterial communities in the experimental site, prior to modulation with the use of feed additives. Also, pre and post natural outbreak infection with pathogenic *Vibrio* spp during *in vivo* trials, where the disease has been confirmed and occurs in regular according to shrimp stage and season, a good comprehension of the shrimp cycle must be included to develop relevant data that lead to beneficial results and scientific report.

Future scientific work will be suggested for optimal control and methodologies under the main aspects of the trial monitoring, 1) feed additives inclusion and dosage 2) feed manufacturing and equipment 3) post larvae selection 4) feed monitoring and feeding trays 5) mortality records 6) weighting 7) samples collection 8) water monitoring (automatization) 9) temperature vs density and 10) staff training prior to trial set up.

A clear comparison with similar conditions must be taken into account in order to extrapolate data and develop models to predict commercial scenarios with economic variables. We should also be examining the role of functional feed additives in association with the increasing practice of generating stable biofloc in intensive shrimp culture. The prebiotic role of many feed additives has been recognised in terms of their use within feeds, but much more scope is possible to use these natural materials to initiate and maintain bacteria in flocculates to improve production. There are enormous possibilities for combined use of such agents with probiotics in pond management to affect the best environmental conditions for intensive shrimp production.

With climate change and increasing temperatures and changing oxygen levels it would seem pertinent to consider such measures in future practice. Although in our studies we mainly addressed the role of feed additives, including β glucan, herbs, and protein hydrolisate, in terms of gut health, it should be noted that such feed additives may also provide functional properties to promote the health of gill tissue and provide a means to enhance the robustness and integrity of the mucosal barrier interface and immune-competence. They may also confer

an antimicrobial function on surfaces including mucus and carapace of shrimp especially during the stressful inter-molt stages. The level of stress encounters in shrimp production is related to many factors. Dominant individuals stress relates to stocking density and also in transport of shrimp that is one of the major husbandry aspects of modern shrimp culture in which major losses can incur. We recommend much more research in this direction to meet legislative standards, hygiene and increasing concerns for animal welfare.

6.2.1. The evaluation of shrimp diets under commercial conditions

Our future work with shrimps around aquatic nutrition will be performed at the site in Colima in between commercial shrimp cycles in where we will have the opportunity to evaluate and perform scientific work will the support of professionals, technicians, students as well scientist from numerous universities around the world. Our work line is to reduce the bridge between academia and commercial needs mainly with the selection of macro and micro ingredients, under changing markets and volatile shrimp prices.

A second project will involve an MSc program with partners universities in where BSc, MSc and even PhD students can visit our site and run their experimental trials with the nurseries tanks recently installed. The quality of work behind these activities will seek support from biotechnology companies, feed mills, farmers who are in the same research line. A comparative between farm conditions in order to make comparisons is fundamental for performance, health and gut presence.

A project will be developed for a small R&D unit, a branch department from Azteca Mills in where new analysis technology can be implemented to develop sustainable and econutritional diets for shrimp performance.

Currently in Mexico, commercial feeding trials with the Republic of China are now becoming more often, when performed by commercial feed companies and tested in commercial scenarios, they have their own R&D facilities for internal nutrition trials for multiple fish and shrimp's species. In order to perform good quality trials a specialized technical team needs to be developed among Mexican students as well foreign interns, while we support the industry to validate their products in multiple aspects, while increasing shrimp production around the country as the main objective, some regions of Mexico will benefit from rural jobs in shrimp farming.

6.2.2. The use of novel feed additives

The use of multiple biotechnologies is becoming a strong tool in aquafeeds as the lack of high-quality ingredients increases, a terrestrial protein source will be a good option for aquafeeds, from essential oils, organic salts, yeast extracts, enzymes, probiotics, peptides and blends will be usefull tools for nutritionists.

In where some meals and fats from fish will arrive mainly from Aquaculture fish rendering (Tacon et al, 2014), with an increase in the use of terrestrials as well, some protein sources

together with nutritional solutions will include a large list of organic compound and mixtures in where specific functionality will be target. Separately, shrimp gut health research will be increasing in multiple aspects including changes related to specific diets and under control testing as well commercial will take place in multiple conditions and locations. Gut bacterial modulation and other organs will be sequenced with better technology (Nano Oxford) that will involve small carry-on devices that will run NGS sequences. One good positive evidence is the price in these sequences and bioinformatics analysis over the last ten years, with a dramatic decrease in prices, bringing new opportunities to increase the knowledge around the gut presence, diversity and interaction with the environment when feed with multiples organic compounds. Lastly from the commercial point of view the Feed additives business will increase their offer of solutions in where aquatic animal nutrition will be around the 5 % of total animal production being aquaculture the fastest sector with a steady growth around 7 % globally (FAO, SOFIA 2018), not to mentioned the new species emerging for aquaculture in where there great potential can be achieve with domestic species around the globe (Tacon et al, 2012) from where more high quality protein will be produced mainly in open oceans and desertic land with high salinity waters.

6.2.3. Life stages and bacteria changes

When analysing larvae stages and the modulation of gut microbiota we actually see main bacterial groups in most scientific documents, starting with most prevalent as Proteobacteria, Bacteroides and Actinobacteria, dominated the intestinal bacteria and the minority Firmicutes

, the real challenge from my own experience is to actually document the modulation of the gut microbiota under multiple production systems with different environments, until then we will be able to create a map of the gut microbiome at different life stages for individual regions. Having a bacterial map will bring a clear of which components benefit specific bacterial communities, in the gut microbiome when feeding on specific organic compounds, and its only after a few analyses at the same site (under similar conditions) in where we could actually see how this microorganism collaborate in similar environments. Lastly some documents suggest that low presence bacteria can actually have an important role in multiple metabolic functions in vertebrates suggesting a sensitive microbiome due to challenges in the environment and nutrition compounds. It's worth to continue to read and analyse data from similar trials to develop a map of what is the mechanism affecting shrimp health, performance and nutrition.

6.2 Overall conclusions

The new systemic paradigm faced by the science of century XXI presents the idea that all knowledge and science must be inter or even transdisciplinary. This organic perspective aims at combine science with ethics in all their aspects. In this context, all science is interconnected, with no space for a reductionist view.

Thus, the study of animal nutrition is linked to animal health, which in its turn is connected to the production system, which is interconnected to the farming process and to the

environment. Therefore, when studying possible new food additives for shrimp farming, we must have in mind the animal welfare and the sustainability of production in both financial and ecological terms.

As already debated, all the substances and preparations discussed in the previous chapters and briefly presented in this chapter showed to be, experimentally, viable to the shrimp farming. Through a science with an organic viewpoint, we demonstrated the use of feed additives, such as yeasts, terrestrial herbs, and tuna liquid hydrolysate, is feasible and practicable in shrimp farming. Their adoption has been shown to be beneficial for animal health and favourable for the production of farmed shrimp, in addition to a contribution to a greener aquaculture agenda.

Additionally, the use of functional feed additives (prebiotics) is recommended to be included before the pathogenic outbreak to activate immune response mechanism in advance as a tool to increase physiological immune response prior to infection or stress situations, again this strategy can only be applied after multiple cycles and with an excellent record keeping and inventories from shrimp ponds.

A multiple functional feed additive can work in synergetic changes of the gut population (microbiota) and the well incorporation of beneficial bacterial in the ponds, where all elements can lead to positive and negative effects.

It’s also important to mention the trend in commercial feed nutrition with the use of functional feeds know as therapeutic diets, still have challenges to overcome. The farmer with high standards needs a solid understanding of the bacterial kinetics on the site, unless the feed company can provide on-site technical support from the feed company as to adequate use of those specific feeds. Otherwise whether the shrimp fitness is good or not and conditions allow the potential from the YAH to be expressed the results will be undervalued.

When performing nutrition with floating cages we must consider some strengths and limitations from this infrastructure, first it’s an affordable solution for students, feed companies and farmers who wish to evaluate feed performance under commercial conditions in the same ponds, because the management required is similar to farming conditions, unfortunately this methodology is limited for health trials and physiological analysis due to limited control.

The intensification and rapid growth in aquaculture activities require stinger control and management measures to avoid the emergence and transmission of diseases. The use of antibiotics it is not a viable solution and an alternative strategy to the use of antibiotics is the application of immunostimulatory substances as dietary supplements, such as glucans. β-glucans, yeast extracts and tunal liquid hydrolyzates have appeared to be convenient for use in aquaculture and have been proved to have a positive effect on the growth and survival of

shrimp, and it also has been shown to enhance healthy microbiota agains pathogenic bacterial and posible viral infections. The modulation of the immune response, improving gut morphology, feed conversion ratio (FCR) growth is a real possibility for organic compounds in Shrimp feeds. A low level of addition and coordinated feeding regime does not affect growth performance in shrimps, in fact, it promotes a higher assimilation of natural food and flow protein from aqua feeds when they reach commercial size lowering FCR and increasing profits for the farmer.

6.4 Summary

The present programme of research sought to contribute to the understanding of the influence of selected functional feed additives on the performance and health of the Pacific white shrimp, *Litopenaeus vannamei*. The analyses described in the scope of this thesis confirm the use of FFA, such as potential modulators of intestinal health in shrimp, immunostimulants. These attributes addressed animal health and feed quality for an economically important aquatic species. Our research may offer significant benefits and profits to shrimp farming under the right conditions. As future prospects, we recommend additional research related to understanding further the complex immune parameters, gene transcription, enzymatic studies and challenge trials required in order to broaden the comprehension of the influence of FFA on shrimp health and performance.

In summary, in order to achieve a more technological, productive and sustainable shrimp farming industry, it is necessary to adopt new preventive health management such as the use of functional feed additives in our portfolio.

In conclusion, the work was undertaken in Mexico, and it is highly relevant to the active shrimp industry in Latin America. With the ever-expanding production of shrimp in Asia and in particular China, it is evident that our work can be taken to provide important novel dietary strategies to mitigate the potential threat of disease such as EMS and White spot as well as emerging pathogens. The cost benefit analysis and societal impact of using functional feed additives must be appraised if this dynamic sector of aquaculture can achieve sustained and economically viable growth to provide safe and quality seafood for the consumer.

7. **APPENDIX**

7.1 Complete DNA extraction protocol

Commercial Kit for DNA extraction, silica membrane-based purification, for samples with high concentration of PCR inhibitors → QIAamp DNA Stool mini Kit, Qiagen®

Equipment → heating block, centrifuge (for 13000 rpm/17000 *g*)

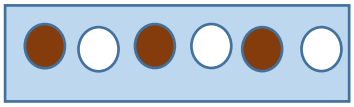
Preparing the samples:

- ✓ If samples are frozen → In order to preserve DNA integrity, defrost the samples slowly, preferably in an ice bath (especially if samples were in -80°C) → do not place in heat source such as dry bath;
- ✓ If samples are fixed in solution →centrifuge for 5 min, 13000 rpm/17000 *g*; after, remove as much as possible of the supernatant;

- ✓ Weight up to 220 mg of sample. It can be less, as 100 mg or even 50 mg. Use sterile material, as sterile tweezers and microtubes. To reuse the tweezer, flambe it after dipping into ethanol;
    - If DNA extraction will take place only next day, it is possible to weight the samples one day before, to save time. If the case, store weighted samples at -20°C, with no other reagent.

P.S.:

- ✓ Duration: ~ 3 hours
- ✓ Work close to a Bunsen burner and with the blue flame to avoid cross-contamination. In an exceptional situation with no Bunsen burner, place samples with a space between them:



- ✓ If some buffer precipitates (buffer ASL or AL, especially), incubate it at around 50°C; mix all buffers before use.

- ✓ The material, besides extraction kit:
    - gloves + labelling pen +lab coat + rack for microtubes
        - Some people prefer to work wearing no gloves, as the risk to burn ourselves exists; I always wear gloves, because I worry about the contamination for myself.

    - sterile tips with filter:
        - 1000 µl
        - 200 µl
        - 20 µl
    - 1.5 ml sterile microtubes (at least 4 per sample)
    - Lysozyme + TE buffer

DNA extraction

Lysis

1. Take the weighted sample and add 500 µl of fresh lysozyme (50 mg/mL in TE 1x); mix *sample + lysozyme*. Incubate 30 min at 37°C.
2. Add 800 µl of Buffer ASL and vortex until totally mixed.
3. Heating block for 10 min at 90°C.
4. Vortex for 15 sec and centrifuge for 2 min, 13000 rpm/17000 *g.*

Inhibitor removal

5. Place 800 µl of supernatant into a new microtube. Add half an Inhibitex tablet. Vortex until suspended. Stand for 1 min.
6. Centrifuge for 3 min. Place 300 µl into a new microtube. Retain the remaining sample frozen, if necessary, to repeat the DNA extraction in the future.

Protein removal

7. Add 20 µl of proteinase K in the collected supernatant.
8. Add 300 µl of Buffer AL. Up and down with the pipette.
9. Incubate for 1 hour at 56 °C.
10. Add 300 µl of cold ethanol 100%. Vortex.

Steps 6 + 7 + 8:
While centrifuge, you can prepare a new microtube with Buffer AL + proteinase K, and after just add the supernatant.

Cold ethanol helps to increase DNA yields. Keep it in the freezer.

Clean-up

11. Apply half (or 500 µl) of the sample to a QIAamp column + collection tube. Centrifuge for 1 min. Discard the liquid. Apply the remaining sample in the same QIAamp column and centrifuge for 1 min again. Discard the liquid
12. Add 500 µl of Buffer AW1. Centrifuge for 2 min. Discard the liquid.
13. Add 500 µl of Buffer AW2. Centrifuge for 3 min. Discard the liquid.
14. Centrifuge again for 1 min, to remove any remaining reagent in the column.
15. Place column into a new microtube and add 50 µl of Buffer AE right in the very middle of the column. This is the elution and final buffer.
    - If you start the DNA extraction with a very low quantity of material, add only 30 µl of Buffer AE.
16. Stand for 5 min. Centrifuge for 3 min. The liquid filtrate contains the extracted DNA. You can discard the column.
17. Store at 4°C up to 1 week, or -20°C for the long term.

Before start:

- ✓ Turn on heating blocks: 37°C, 56°C, and 90°C;

- ✓ Clean the bench with Virkon solution/ethanol 70°;

Lysozyme calculation

TE buffer 1x → it normally comes in the concentration of 100x

1 part of TE 100x : 99 part of molecular grade water

1 ml TE 100x : 99 ml of molecular grade water

50mg of lysozyme / 1ml TE 1x

500 µL /sample

For example, if 16 samples:

500 µL of lysozyme x 16 samples = 8ml of lysozyme in final volume

50mg/ml → 50mg x 8ml TE 1x = 400mg of powder lysozyme

For 16 samples: 400 mg of powder lysozyme + 8 ml TE 1x

P.S. Always calculate for one or two additional samples, to have a margin and not work in the limit.

DNA quality

Checking DNA Quality and Quantity (after DNA extraction)

Equipment → NanoDrop or similar

P.S.:

- ✓ This is not a mandatory step, but it gives DNA quality and quantity;
- ✓ It is expected to extracted DNA presents high quality. If not, do not despair and try the PCR even so; the primer pair is excellent and the chance to amplify is high;
- ✓ DNA quantification is variable.

DNA quantification:

- ✓ Optimal DNA template concentration used for PCR depends on the primer pair. Commonly, it is used 1 ng/µL or 100 ng/µL;

- ✓ In the case of primer pair 27F & 338(I+II) R, for 16S, it amplifies low DNA concentration, as 1 ng/µL;
- ✓ You can standard the samples all to 1-10 ng/µL, and use the standardized templates;
- ✓ DNA extraction with commercial kits usually results in lower quantification, less than 100 ng/µL.

Calculation for dilution:

C1 x V1 = C2 x V2, where:
C1: DNA concentration gave by NanoDrop, in ng/µL
V1: DNA volume, in µL, that I need to use to achieve the final concentration I want to, the "X"
C2: final DNA concentration that I want to reach, in ng/µL
V2: total final volume, in µL, that I want to have in the microtube (water + DNA)

Example → Quantification measured by NanoDrop: 36 ng/µL
Concentration I want to reach: 10 ng/µL
Total final volume that I want to have: 20 µL

36 ng/µL x "X" = 10 ng/µL x 20 µL
"X" = 5,5 µL of extracted DNA
20 µL – 5,5 µL = 14,5 µL of ultrapure water
At the end: 5,5 µL of extracted DNA + 14,5 µL of ultrapure water in a new microtube

Using NanoDrop™ 2000 Spectrophotometer, ThermoFischer
Material:

- ✓ Pipette + tips for 2 µL
- ✓ Buffer AE (the same used for DNA elution in DNA extraction, the last buffer)
- ✓ Extracted samples

1. Open the corresponding software
2. Choose the option: Nucleic Acid – DNA (not protein, not cDNA, not RNA)
3. Clean the sensor with soft paper
4. Put 2 µL of ultrapure **water** in the sensor. Close it. Press **blank**.
5. Clean the sensor with soft paper
6. Homogenize the extracted DNA. Put 2 µL of the **sample** in the sensor, avoiding bubbles. Press **measure**.
7. Clean the sensor with soft paper
8. Put the next sample.

9. Go on, always cleaning between samples. If you have a huge number of samples to quantify, make another blank with ultrapure water whenever you think it is necessary.

During quantification, take notes of:

- ✓ DNA quantification; it will appear in ng/µL
- ✓ Graph curve: it should have one high peak
- ✓ 260/280 relation (DNA/protein): it should be ~ 1.8. If too low, maybe the sample have contaminants, as phenol or proteins.
- ✓ 260/230 (DNA/salt): it should be ~ 1.8 – 2.2.
    - ✓ If the numerical quality is not ideal, try the PCR even so. It can amplify.

7.2 PCR 16S

DNA amplification

Source: Gajardo, K., Rodiles, A., Kortner, T. M., Krogdahl, Å., Bakke, A. M., Merrifield, D. L., & Sørum, H. (2016). A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*): a basis for comparative gut microbial research. *Scientific reports*, *6*, 30893.

Duration:

- ✓ Reaction assembly: ~ 20 – 30 min, depends on the number of samples + UV time
- ✓ PCR reaction (thermocycler): ~ 1h 40min

Equipment:

- ✓ Centrifuge
- ✓ Laminar flow cabinet
- ✓ Microcentrifuge
- ✓ Thermocycler

Material:

- ✓ Pipette + filter tips for 2 µL; 20 µL; 200 µL; 1000 µL
- ✓ Microtubes: 200 µl; 600 µl; 1,5 µl
- ✓ Gloves + rack for microtubes
- ✓ PCR reagents
    - Taq mix (MyTaq™ Red MiX; Bioline®)
    - Primer Pair (25 pMol – use concentration) → with the reverse primer, after dilution, mix the R-I and R-II in the same proportion and use this mix; with the forward primer, dilute and use it.
    - Molecular grade water
    - Extracted (and diluted) DNA
    -

Primer pair:

27F (5’ AGA GTT TGA TCM TGG CTC AG 3’)

338R: a pool of R-I and R-II

338R-I (5’ GCW GCC TCC CGT AGG AGT 3’)
338R-II (5’ GCW GCC ACC CGT AGG TGT 3’)

- ✓ Optimal DNA concentration: 1ng.μl^{-1}
- ✓ Amplification: 16S rRNA gene, from V1-V2 regions
- ✓ Expected size: 350 bp (confirmed on agarose gel)

PCR Conditions & Cycles

Reagents	Volume
Taq mix (MyTaq™ Red MiX; Bioline®)	25 µl
Primer Forward (25 pMol)	1 µl
Primer Reverse (25 pMol)	1 µl
Molecular water	22 µl
49 µl of mix + 1µl of extracted DNA	

Example: for 15 samples + 1 negative control + 1 positive control = 17 samples + 2 margins = calculation for 19.

Reagents	Reaction Volume (1x)	Total volume (x19)
Taq mix	25 µl	475 µl (25 x 19)
Primer Forward	1 µl	19 µl (1 x 19)
Primer Reverse	1 µl	19 µl (1 x 19)
Molecular water	22 µl	418 µl (22 x 19)
Total	49 µl	931 µl

Phase	Temperature (°C)	Time	Cycles
Initial denaturation	94	7’	
Denaturation	94	30”	10 x touchdown
Hybridization	63 - 53	30”	
Elongation	72	30”	
Denaturation	94	30”	25 x
Hybridization	53	30”	
Elongation	72	30”	
Final Elongation	72	10’	
Finalization	10	Until end	

If PCR does not amplify, you can try to:

1. Instead of 53°C in hybridization, it can be tried 55°C;
2. Modify the volume of DNA, as with 0.5, 2, 3 or 5 μl. If so, adjust water volume in the PCR reaction mix calculation.
    a. For example, with 3 μl extracted DNA + 47 μl reaction mix (total volume 50 μl; 20 μl of molecular grade water).

Step by step

Preparation

1. Clean the inside of the cabinet; clean the material, as pipettes and racks.
    a. If the cabinet is made of plastic, clean only with chlorine
    b. If the cabinet is made of glass, you can use ethanol 70°
2. Put inside the cabinet:
    a. Gloves
    b. Labelling pen
    c. Microtubes
    d. Molecular water
    e. Pipettes
    f. Rack for microtubes
    g. Tips
3. Close the cabinet. Turn on UV light for 20 min. After turn off, wait some minutes to allow ozone gas to dissipate.
4. Keep close the microcentrifuge

Reaction assembly

1. Spin the extracted DNA. Spin primers and reagents;
2. After calculating the final volume of the reaction mix, do it;
3. Distribute 49 μl of the reaction mix to each PCR microtube (200 μl);
4. Pipette 1 μl of each sample in the corresponding microtube + negative control (molecular water);
    a. You can pipette 1 μl in the microtube wall, to check visually if the tiny ball is there;
    b. You can pipette 1 μl inside the liquid and up-and-down to be sure the tiny volume is out.
5. Pipette 1 μl of the positive control (last sample to work with)
    a. A positive control can be a bacteria grown in liquid medium, not necessarily extracted

Put the microtubes with *reaction mix + samples* in the thermocycler. Turn on the corresponding program.

- ✓ You shoul*d wear a separated lab* coat only for PCR reactions. Do not wear the same lab coat that is used for microbiology, for example.
- ✓ There are some labs that have a separated room only for assembly of PCR reactions. In this case, you can wear no lab coat. In this case, is also possible to do the PCR reaction out of the cabinet, as the entire room is separated only for this purpose.
    - As this PCR is to amplify 16S, it is always preferable to use a cabinet, as the easy contamination.
- ✓ <u>Important</u> → In order to get enough DNA to be sequenced, probably it will be necessary 2 or 3 PCR amplifications to purify and go forward. *Or*, it is also possible to double all PCR volumes and do one reaction with 100 μl (instead of 50 μl). In the end, put all the PCRs products together (of the same sample!) in the same microtube and move on for the purification step. This step is important especially if the band is too weak in the agarose gel, which indicates, indirectly, that the amount of amplified DNA was small.
- ✓ If the negative control shows contaminated (with a band in the agarose gel), do all the PCR reaction again. The reaction is contaminated, and it is invalidated.
    - Take care with the molecular grade water used in the PCR reaction. Keep it separated and only use the separated aliquots/bottle to PCRs.

7.3 PCR product purification

Commercial Kit → Agencourt AMPure XP, Magnetic Beads, Beckman Coulter®
+ Magnetic rack for separation of magnetic beads

P.S.:

- ✓ Work close to a Bunsen burner and with the blue flame to avoid cross-contamination;

Material & Equipment:

- ✓ 1.5 ml microtube (at least 2 per sample)
- ✓ Common microtube rack
- ✓ Gloves
- ✓ Lab coat
- ✓ Labelling pen
- ✓ Micro centrifuge
- ✓ Molecular grade pure ethanol
- ✓ Pipette + filter tips for 200 µL and 1000 µL
- ✓ Pipette with big capacity, as the serological one
- ✓ Ultrapure water

Preparation:

- ✓ Spin the samples (PCR products); it is not necessary to purify any control;
- ✓ With a pipette, measure the total volume inside the microtubes of the samples. Probably they will all have the same volume.
  - Place all its contents to a new 1.5 ml sterile microtube (it came from the PCR in a 200 µL microtube);
- ✓ Calculate how much Ampure XP reagent will be used per sample:
  - "X" x 0.8
  - If the total volume of the sample is 45 µL → 45 µL x 0.8 = 36 µL/sample
- ✓ Prepare ethanol 70%:
  - 800 µL per sample
  - If 12 samples → 12 x 800 µL = 9.6ml → prepare 10 ml of ethanol 70%
    - 7 ml molecular grade ethanol 100%
    - 3 ml ultrapure water
- ✓ Clean the bench with Virkon solution/ethanol 70°;

Purification Steps

Attaching amplicons and magnetic beads

1. Vortex the AMPure XP reagent;
2. Add AMPure XP reagent to the *1.5 ml microtube with the sample*; in the example above, add 36 µL per sample; Up and down five times.
3. Wait 5 min with the microtube in a common rack; no vortex, no centrifuge;

Separating amplicons from contaminants

4. After waiting 5 min, place the *microtube with the samples* in the special magnetic rack; wait until the beads stick to the magnetic plate. The solution will become clear (~ 2 – 3 min).

Washing

5. Still in the magnetic rack;
6. With care and a pipette + tip, remove the liquid, leaving only the beads in the microtube wall; avoid pulling the beads; close the microtube to not dry the samples;
7. Add 400 µL of ethanol 70%, washing the tube wall;
8. Still in the magnetic rack, rotate the microtube 360° 2 times to the right, and 360° 2 times to the left, as illustrated in the figure above:

9. Remove the ethanol with pipette + tip. Remain the beads.
10. One more time, add 400 µL of ethanol 70%, washing the tube wall;
11. Rotate the microtube 360° 2 times to the right, and 360° 2 times to the left;
12. Remove the ethanol with pipette + tip. Remain the beads;
13. Open all *microtubes with the samples* close to the flame and wait until all the ethanol evaporates;
    - Maximum 5 min. If remains small droplets, they are only water – not a problem.
    - If the rack has samples in both sides, turn it in the middle time to dry equally.

Amplicon elution

14. Add 30 µL of molecular grade water right above the beads; do not mix, do not vortex;
15. Place the microtubes in a common rack (remove from the magnetic rack); wait 3 min;
    - Do not vortex, do not mix
    - The beads will release the DNA, which will come in solution to the water
16. After 3 min, replace the microtubes to the magnetic rack; wait 1 min;
    - The beads will stick to the magnetic plate, but the DNA will remain in solution

17. Still in the magnetic rack, with care and a pipette + tip, transfer the eluted DNA (liquid without the beads) to a new sterile microtube;
    - Pull 20 µL and after the rest, to avoid pulling the beads together;
    - The original microtube with the beads remain in the magnetic rack, otherwise, the beads will mix with the eluted DNA again
    - If the beads and DNA mix again (liquid become dark), put the microtube with the samples in the magnetic rack and wait until they separate again (liquid become clear)
18. Close well and store at -20°.

P.S.:

- ✓ From now on, the sample is purified and ready to go to sequencing process;
- ✓ It may be necessary to quantify the samples after purification. It is similar to the NanoDrop procedure, but the most indicated equipment is the Qubit Fluorometer because it is more precise;
- ✓ This protocol presents some adaptations (volume/time) in relation to the protocol indicated by the manufacturer/kit. Both protocols work well.

7.4 Agarose Gel

Agarose Gel Electrophoresis

Equipment:

- ✓ Electrophoresis apparatus (power supply + tank + casting plate + comb)
- ✓ Microwave
- ✓ UV light transilluminator

Material & Reagents:

- ✓ Amplified DNA (PCR product)
- ✓ Becker
- ✓ Buffer – TAE or TBE
- ✓ Distilled water
- ✓ DNA ladder (size marker) of 100 bp
- ✓ Dye – Gel Red, Syber Safe
- ✓ Graduated cylinder (50ml / 100ml)
- ✓ Molecular grade agarose
- ✓ Pipette and tips for 20 µl

1.5% agarose gel

1. Calculate the necessary volume for the gel, according to the size of your tank;
2. Mix TAE 1x + agarose in a Becker. In the microwave, boil until the agar dissolves; Swirl to resuspend any precipitated agar; boil again if necessary. Cool until it reaches a temperature you can hold the Becker in your hands;
3. Add 1 µl of Syber Safe for every 10 ml of agarose gel;
    a. For example: 60ml TAE 1x + 0.9 ml agarose. Add 6 µl of Syber Safe.
4. Pour the gel mixture into the plate. Put the comb. Wait until the agar has solidified.

Loading the samples

1. Spin the samples.
2. Remove the comb and place the *gel + plate* inside the tank. Cover *gel + plate* with the same buffer concentration you used before (TAE 1x);
3. Apply the whole content of the DNA ladder in the first well;
4. Apply 5 µl of each sample in each well. One tip per sample.
    a. Suggestion: ladder – positive control – samples – negative control – ladder again
5. Connect the electrode leads to the power supply. Run for ~ 40 min, at 80V – 100V.
    a. Do not forget the power supply on with the gel inside, otherwise, samples will leave the gel
6. Disconnect the electrode leads. Visualize the gel in a UV light transilluminator.

P.S.: If you want to do check the quality of the extracted DNA in an Agarose Gel, just adjust the percentage and time: 0.8 – 1.0% agarose gel, for 1h – 1h30min.

7.5 Statistics data from performance trials, Chapter 3

DATA FROM TRIALS

Cages / Treatments	N WEIGHT	Mín	Máx	S WEIGHTS	Media	Desv. típ. STDEV
Control WHITEC1	47	13	24	949	20.191	2.071
Control WHITEB3	44	14	28	807	18.341	2.579
Control WHITED3	56	14	23	1,083	19.339	2.160
Control WHITEA5	27	14	24	544	20.148	2.214
ExCell GREENA1	71	13	24	1,425	20.070	2.134
ExCell GREENB1	59	16	25	1,256	21.288	2.018
ExCell GREENC4	66	15	21	1,189	18.015	1.554
ExCell GREENC5	65	18	26	1,399	21.523	1.969
B-S BLUEC2	60	18	27	1,309	21.817	1.970
B-S BLUED2	59	15	24	1,130	19.153	1.901
B-S BLUEB4	55	13	23	931	16.927	2.107
B-S BLUED4	50	13	21	877	17.540	1.940
B-S Plus REDD1	69	12	22	1,172	16.986	2.033
B-S Plus REDB2	63	12	23	1,116	17.714	2.406
B-S Plus REDA4	67	14	23	1,278	19.075	2.077
B-S Plus REDD4	66	16	23	1,286	19.485	1.712
3 Component YELLOWA2	77	14	21	1,357	17.623	1.358
3 Component YELLOWA3	68	12	23	1,242	18.265	2.085
3 Component YELLOWC3	73	13	21	1,230	16.849	2.093
3 Component YELLOWB5	68	15	23	1,285	18.897	1.788
N valid	27					

7.6 FCR and SGR Statistics, Chapter 3

Treatment	Initial biomass [g]	Final biomass [g]	Real biomass [g]	Total feed [g]	Period of time [days]	FCR	Average FCR	FCR SD	SGR	Average SGR	SGR SD
Control (white)	298	949	651	2,456	143	3.773	4.971	2.815	0.810	0.690	0.219
Control (white)	311	807	496	2,129	143	4.292			0.667		
Control (white)	303	1,083	780	2,135	143	2.737			0.891		
Control (white)	310	544	234	2,125	143	2.081			0.393		
ExCell (green)	302	1,425	1,123	2,896	143	2.579	2.826	0.241	1.085	1.027	0.048
ExCell (green)	310	1,256	946	2,948	143	3.116			0.978		
ExCell (green)	285	1,190	905	2,644	143	2.922			0.999		
ExCell (green)	314	1,399	1,085	2,915	143	2.687			1.045		
B-S (blue)	290	1,309	1,019	2,504	143	2.457	3.232	0.808	1.054	0.874	0.144
B-S (blue)	304	1,129	825	2,224	143	2.696			0.918		
B-S (blue)	298	931	633	2,254	143	3.561			0.797		
B-S (blue)	310	877	567	2,389	143	4.213			0.727		
B-S Plus (red)	301	1,172	871	2,459	143	2.823	2.728	0.207	0.951	0.967	0.029
B-S Plus (red)	291	1,120	829	2,404	143	2.900			0.942		
B-S Plus (red)	321	1,277	956	2,323	143	2.430			0.966		
B-S Plus (red)	304	1,286	982	2,711	143	2.761			1.009		
3 component (yellow)	300	1,357	1,057	2,670	143	2.526	2.603	0.153	1.055	1.002	0.036
3 component (yellow)	306	1,241	935	2,400	143	2.567			0.979		
3 component (yellow)	302	1,231	929	2,315	143	2.492			0.983		
3 component (yellow)	311	1,285	974	2,755	143	2.829			0.992		

7.7: Ion Torrent runs and samples weight, Chapter 4.

SAMPLE #	CAGE CODE	Ion-Xpress Code	KS CODE	VOLUME
1	C11S2	26	BLUE	30 µl
2	C11S4	27	BLUE	30 µl
3	C6S1	28	BLUE	30 µl
4	C6S5	29	BLUE	25 µl
5	C4S3	30	BLUE	25 µl
6	C4S2	31	BLUE	30 µl
7	C1S3	32	BLUE	30 µl
22	C1S2	-	BLUE	30 µl
8	C5S5	33	RED	25 µl
9	C5S2	34	RED	25 µl
10	C12S5	35	RED	30 µl
11	C12S6	36	RED	30 µl
12	C7S5	-	RED	30 µl
23	C3S4	38	RED	30 µl
30	C3S1	39	RED	30 µl
31	C7S6	46	RED	30 µl
24	C9S1	39	GREEN	25 µl
25	C10S8	40	GREEN	30 µl
26	C10S2	41	GREEN	30 µl
27	C9S4	42	GREEN	30 µl
28	C8S6	43	GREEN	30 µl
29	C8S1	44	GREEN	30 µl
32	C2S5	-	GREEN	30 µl
33	C2S6	47	GREEN	25 µl

7.8 Feeding table at 100 PL/m^2

Stage	Days	Weight (g)	Weight increase (g)	Survival (%)	Percentage of biomass (%)	Week ($kg.m^2$)
Nursery	1	0.01		100	20	20
	7	0.1	0.09	98	15	14.7
	14	0.4	0.3	96.5	12	11.58
Trials	21	1	0.6	95	10	9.5
	28	1.7	0.7	93.5	7	6.55
	35	2.5	0.8	92.5	5.5	5.09
	42	3.3	0.8	91	4.5	4.1
	49	4.4	1.1	90	4	3.6
	56	5.5	1.1	89	3.5	3.12
	63	6.6	1.1	87.5	3	2.63
	70	7.8	1.2	86.5	2.5	2.16
	77	9	1.2	85.5	2	1.71

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